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TOXICITY OF DDT FOOD AND WATER EXPOSURE TO FATHEAD MINNOWS

Environmental Research Laboratory
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FOREWORD

Our nation's freshwaters are vital for all animals and plants, yet our diverse uses of water---for recreation, food, energy, transportation, and industry---physically and chemically alter lakes, rivers, and streams. Such alterations threaten terrestrial organisms, as well as those living in water. The Environmental Research Laboratory in Duluth, Minnesota develops methods, conducts laboratory and field studies, and extrapolates research findings

--to determine how physical and chemical pollution affects aquatic life

--to assess the effects of pollutants

--to predict effects of pollutants on large lakes through use of models

--to measure bioaccumulation of pollutants in aquatic organisms that are consumed by other animals, including man

This report determines the effects of DDT on fathead minnows when they are exposed to it in the food and/or water.

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ABSTRACT

Fathead minnows (Pimephales promelas) were exposed during a partial chronic toxicity test to two DDT concentrations in the water, one in the diet, and combinations of water and diet for 266 days through a reproductive period of their life cycle. Tissue-residue analyses were performed on test fish at preset intervals throughout the exposure and also on embryos, larvae at hatch, and 30- and 60-day progeny. The contribution of DDT from each source was monitored with gas-chromatography and liquid-scintillation techniques. The diet was clams that had accumulated 14C-DDT when exposed at a DDT water concentration similar to that in the high fish exposure.

Higher total DDT tissue residues were accumulated from the water than from the diet. Residues contributed by dietary DDT were additive to those from the water. Mean concentration factors were 1.2 times from the diet and 100,000 times from the water. Mortality was higher in fish exposed to DDT in both water and diet than in fish exposed to only one or the other of these sources. DDT in the diet significantly reduced the probability of survival of the test fish (P=0.025). Estimated maximum acceptable toxicant concentrations for DDT are 0.9 µg/l for fish exposed to DDT in the water only or 0.4 µg/l for fish exposed to DDT in both water and diet. Embryo DDT residues and larval mortality were about twice as great for embryos and larvae from parent fish that had been exposed to DDT in both water and diet as for those from parent fish exposed to DDT in the water only.

About 60% of the mean total micrograms of combined DDT analogs in fish that had been exposed to DDT at 0.5 µg/l in the water and in the diet was eliminated within 56 days. Almost all of the eliminated DDT was dietary DDT. Elimination in fish that had been exposed to DDT in the water only was negligible.

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ACKNOWLEDGMENTS

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SECTION I

INTRODUCTION

There are many opinions among aquatic researchers about the importance of water concentration and food chain as sources for biological magnification of pesticides in the aquatic environment. Some authors suggest the food chain as the major source (Macek and Korn, 1970; Harrison *et al.*, 1970; Johnson *et al.*, 1971; Eberhardt *et al.*, 1971), whereas others (Reinert, 1967, 1970; Chadwick and Brocksen, 1969; Grzenda *et al.*, 1970; Murphy, 1971; Hamelink *et al.*, 1971; Epifanio, 1973) stress the water concentration. No data are available indicating the relationship of either of these views to those situations where both sources are involved at threshold levels of chronic toxicity.

Current pesticide standards are based upon water concentration alone. We must also know what effect the presence of pesticide-laden food-chain organisms has on aquatic life so that accurate pesticide standards can be developed. The following study was initiated in response to this problem. The objectives were to determine whether DDT accumulation in fathead minnows (*Pimephales promelas*) is more affected by a food or water source, to determine whether persistent pesticide exposure through both food and water is more toxic (or creates higher residues) than exposure through only one or the other of these routes, to estimate a partial chronic maximum acceptable toxicant concentration for DDT, and to determine DDT concentration factors for fathead minnows and freshwater clams used as the food.

SECTION II

CONCLUSIONS

Fathead minnow DDT concentration factors are about 1.2 times from the diet and 100,000 times from the water. Clams used as the DDT food source had a magnification factor of about 25,000 times when exposed to a similar water concentration (2.0 µg/l). Tissue residues in fish exposed to dietary DDT only were about one-fourth as high as residues in fish exposed to a water concentration equal to that at which the food had been exposed. Dietary DDT tissue residues were additive to those resulting from DDT water concentrations.

Fathead minnow mortality was greater in fish exposed to DDT through both food and water than in those exposed through only one or the other of these routes. Water exposure alone, however, was more toxic than food exposure alone. Mortality results agree closely with those for residue accumulation, indicating that higher mortality occurs with higher mean tissue residues.

An estimated maximum acceptable toxicant concentration for this test is 0.9 µg/l for water exposure alone or 0.4 µg/l when DDT is present both in water and in diet (45.6 µg/g).

Elimination of DDT from fathead minnows that had been exposed to it in the water at 0.5 µg/l or at the same water concentration and in the diet also indicated that about 60% of the accumulated mean total micrograms of total DDT in fish that were exposed to it in diet and in the water was eliminated within 56 days. Elimination from fish exposed to DDT in the water only was negligible. It appears that there is a selective mechanism for elimination of dietary DDT.

Exposure of fish to DDT in diet or in water are both important and should be considered together in future studies. Presence of dietary DDT can reduce the maximum acceptable toxicant concentration.

SECTION III

RECOMMENDATIONS

It is recommended that chronic toxicity studies on persistent toxicants be performed with additional consideration for possible accumulation through the food chain. More such studies are needed to evaluate the combined food and water toxicant effect on fish and other aquatic life. Future studies should be designed to provide food-effect data needed to derive more refined criteria necessary to determine the survival requirements for aquatic life.

SECTION IV

MATERIALS AND METHODS

The basic design of the partial chronic exposure followed the recommended procedures set forth by the National Water Quality Laboratory Committee on Aquatic Bioassays (Appendix).

PHYSICAL CONDITIONS

Fathead Minnow Exposure

A proportional diluter (Mount and Brungs, 1967) was modified to deliver two test concentrations and a control with the low test concentration one-quarter that of the high concentration. The toxicant was introduced by a 50-ml injector syringe with a Teflon® needle from an acetone stock solution. The syringe was calibrated to inject 8.7 µl of stock solution per cycle. The highest level of acetone ever reached, within any 24-hr period, in the high concentration test chamber was 5 mg/l. Nominal DDT test concentrations, selected on the basis of acute and preliminary partial chronic data, were 2.0 and 0.5 µg/l, respectively.

The test water was sand filtered Lake Superior water sterilized with ultraviolet light and warmed to approximately 25° C by a coiled stainless steel heat exchanger located in a stainless steel headbox. A thermoregulator relay system (Syrett and Dawson, 1972) activated tandem solenoid valves that controlled the flow of hot water through the heat exchanger.

The test chambers used for adult exposures measured 91 x 30 x 30 cm and held a water volume of 55 l. Approximately 3 months after the start of the test the adult tank was separated into two sections by stainless steel screen. Two 30.5 x 13.5 x 31.5 cm larval chambers were placed in the back section of each adult chamber, and flow rates were adjusted to provide 250 ml of test water to each larval chamber and 500 ml to the adult

section. The flow of water to the test chambers was adjusted to maintain dissolved oxygen levels at greater than 65% saturation and to provide a 99% replacement time of the test water in each test chamber within 10 hr as determined from Sprague (1969).

Chambers were siphoned daily 1-2 hr after feeding to remove leftover food and were brushed and siphoned weekly.

The photoperiod followed the normal daylight hours of Evansville, Indiana, except that it was necessary to extend the peak photoperiod of 15 hr 45 min for approximately 6 weeks to insure enough larvae for gas-chromatography and liquid-scintillation analysis of tissue residues. Daytime light intensity varied from 25 to 41 ft-c in the adult chambers.

Clam Exposure

The clam exposures were conducted in a flow-through system. The system consisted of a stainless steel headbox with coiled stainless steel heat exchanger and thermoregulator relay system. The test water flowed from the headbox through a solenoid valve to a 24.5 x 13.5 x 16.5 cm water cell. When a predetermined volume was reached, the water siphoned through an inverted u-tube into a 19.5 x 17.5 x 13.0 cm toxicant chamber. Action of the siphoning water flowing into a cup, mounted on an arm, activated a microswitch to shut off the water flow from the headbox and also activated a 50-ml injector syringe with a Teflon® needle to inject 8.7 µl of 14C-DDT-acetone stock solution into the toxicant chamber for a nominal DDT concentration of 2.0 µg/l. The test water then passed through a standpipe siphon into a common 28.0 x 14.7 x 31.0 cm glass chamber where the toxicant and test water were mixed. Water from this chamber flowed through two standpipe siphons to duplicate 152.5 x 30.5 x 28.0 cm stainless steel exposure chambers. Water volume in each chamber was regulated by a standpipe at 74 l. The flow-through apparatus delivered 3.2 l per cycle or 1.6 l per chamber with a 99% replacement of the test water in about 6 hr as determined from Sprague (1969).

BIOLOGICAL CONDITIONS

Fish

On September 13, 1972, one hundred 45(±3)-day-old fathead minnows were randomly assigned to each of 12 test chambers. The 12 chambers were used to expose the

fish through a reproductive period of their life cycle to duplicates of (1) a control; (2) DDT-exposed food, but no DDT in the water; (3) unexposed food, but 0.5 µg/l DDT water exposure; (4) DDT-exposed food and 0.5 µg/l DDT water exposure; (5) unexposed food, but 2.0 µg/l DDT water exposure; and (6) DDT-exposed food and 2.0 µg/l DDT water exposure. The fathead minnows were fed chopped and ovendried clam tissue which was either clean or contaminated by ^{14}C-DDT.

The fish were sampled to determine total body tissue residues at 7, 14, 28, 56, 112, 224, and terminally at 266 days exposure. The fish were not fed for 24 hr before samples were taken. Samples consisted of 10 fish per duplicate test chamber at 7 days of exposure and 5 fish per duplicate thereafter, until terminally, when all remaining fish except those used for an elimination study were sampled. Samples were placed in preweighed glass vials, reweighed, and frozen at -8° C until analyzed. Results were determined both on a whole body wet weight and lipid basis.

At the termination of the 266-day partial chronic exposure 20 adult fathead minnows (10 from each duplicate chamber) were transferred from the 0.5-µg/l DDT water exposure and the 0.5-µg/l DDT water and DDT-contaminated food exposure to separate control chambers for use in an elimination study. Five fish were removed for tissue-residue analysis from each of these chambers at 7, 14, 28, and terminally at 56 days. To prevent bias caused by weight changes, results were determined on a total microgram basis.

During the spawning period embryos in excess of the 50 required for hatchability studies were placed in preweighed glass vials, reweighed, preserved with petroleum ether to prevent dehydration, and stored in a freezer at -8° C. Individual test-chamber samples were composited to provide a minimum of four 0.3-g (about 300 embryos) samples for residue analysis.

Larvae at hatch were transferred in groups of 40 each to larval chambers for 30- and 60-day growth, mortality, and residue studies. The fish were photographed at 30 and 60 days by the method of Martin (1967) as modified by McKim and Benoit (1971) for growth determination. Tissue residues were analyzed for two 30- and one 60-day samples for each of the 12 test chambers.

Larvae at hatch not saved for 30- and 60-day studies were weighed and grouped by the same method used for embryo-residue samples to provide a minimum of two 0.3-g (about 600 larvae) samples per test chamber for residue analysis. Residue analysis on larvae at hatch and embryos was performed only on a whole-body wet-weight basis.

Clams

Chopped, ovendried clam tissue was used as the food source. Clams were chosen because they have a nearly average accumulation factor when compared to other invertebrates (Johnson et al., 1971; Eberhardt et al., 1971), were readily available, are well suited to laboratory conditions, provide a large bulk of storable tissue, and attain equilibrium with DDT in a relatively short time as was indicated in preliminary tests. Five species of clams were collected from the Eau Claire River in Wisconsin: Lampsilis siliquoidea, Lampsilis ventricosa, Lasmigona costata, Fuscoraia flava, and Liqumia recta. The clams were held before DDT exposure in a fiberglass tank through which lake water flowed. Four separate 8-week clam exposures to ^{14}C-DDT were conducted, as preliminary studies indicated that an 8-week exposure period was necessary for the clams to achieve an equilibrium with an exposure concentration of 2.0 µg/l DDT (the same DDT concentration as in the higher fish water exposure).

Clams were placed in the exposure chambers and slowly acclimated to 20° C. Fifty clams were used in each duplicate chamber per exposure, and 100 were held in a fiberglass control chamber through which lake water flowed from the same headbox as the exposure system. The control chamber had a volume of 170 l and a water flow rate to provide 99% replacement in 6 hr. Dissolved oxygen concentrations never dropped below 80% saturation.

The clams were fed daily with a commercial fish fry food and plankton. The flow-through apparatus was monitored daily, the chambers were siphoned every other day, and the sides of the chambers were scrubbed whenever algal or fungal growth became excessive.

After completion of an 8-week exposure the soft parts of the clams were removed, chopped in a blender, and ovendried for 1 1/2-2 hr at 110° C. The dried clam meat was supplemented with a vitamin and mineral mix. A list of ingredients for the mix was obtained from the Fish-Pesticide Research Laboratory, Columbia,

Missouri (Mehrle, personal communication). The prepared food supply was kept frozen, and a small portion was removed daily for feeding.

CHEMICAL CONDITIONS

Fish

Water temperatures were maintained between 24.0 and 25.5° C and were checked daily in all test chambers. Routine water chemistries were determined weekly by the methods described by the American Public Health Association et al. (1971). Dissolved oxygen levels were never lower than 5.4 mg/l nor higher than 8.2 mg/l. Mean total hardness, acidity, and alkalinity were 43.9, 2.8, and 42.5 mg/l, respectively, and were similar to those mentioned by Hermanutz et al. (1973); pH was between 7.2 and 7.8. DDT stock solutions were prepared with DDT, Technical grade (p,p' isomer 77%)* and DDT concentrations in the water were measured once a week. In each sample set, analyses were made on a duplicate and a spiked sample of control water. DDT was extracted from the test water with petroleum ether and analyzed by gas chromatography. Percentage recovery from the spiked control water samples ranged from 38 to 115%; mean recovery was 86.9±3.4% (n=39).

Clams

P,p' DDT ring-UL-14C in benzene with a specific activity of 3.85 µc/mM was procured in 50-µc lots.+ The benzene was evaporated under a stream of nitrogen, and the p,p' DDT ring-UL-14C was redissolved in 50 ml of acetone. To prepare the 14C-DDT stock solution a calculated gram weight of DDT (Tech.) was dissolved in acetone to which 45 ml of the 14C-labeled DDT in acetone was added. Total volume was brought to 250 ml with acetone and thoroughly mixed. The clams were exposed to 2.0 µg/l 14C-DDT nominal concentration and 1.35 µc of 14C per day, or about 76 µc of 14C per 8-week exposure. The 14C-DDT water concentrations ranged from 1.05 to 2.60 µg/l, with a mean concentration of 1.81±0.13 (n=14). Percentage recovery of spiked control water samples ranged from 80 to 121%; mean recovery was 102.6±8.5% (n=5). Water analysis for DDT was performed by the same method as for the fathead minnow exposure.

*DDT (Tech.) was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

+P,p' DDT ring-UL-14C was purchased from Mallinckrodt/Nuclear, St. Louis, Missouri.

RESIDUE ANALYSIS

Gas Chromatography

Extraction of adult fish and 30- and 60-day progeny was accomplished by transfer of the samples to an Eberbach Semi-micro or Micro Container explosion-proof blender (depending upon sample weight). Anhydrous Na_2SO_4 was added to dry the tissues and insure homogenization. Samples were blended with petroleum ether for 2 min at high speed. The solvent extract was decanted onto an anhydrous Na_2SO_4 column and collected in a tared beaker for lipid determination. Each sample was extracted three times. The solvents were evaporated, and lipid weight was determined. The lipids were redissolved in petroleum ether and then cleaned up on a 20-g florisil column. Samples were eluted with 200 ml of 6% ethyl ether/petroleum ether as described by the U.S. Department of Health, Education, and Welfare (1971). Samples were concentrated to a volume of less than 10 ml on a steam bath. Analysis was completed by gas chromatography. Peak heights were measured individually for DDT, DDE, and TDE. DDT and metabolites were then summed to obtain the total DDT present. DDT, DDE, and TDE are expressed as the sum of the orthopara and parapara fractions found in the analyses.

Samples of embryos and larvae at hatch were homogenized in 10 ml of petroleum ether in a glass tissue-grinding tube by using a teflon pestle. Samples were extracted five to eight times depending upon total sample weight and were concentrated as previously described for the adult fish. No cleanup was necessary and no lipid analysis was performed. Residue analysis was performed as for adult fish. Residue analysis on the clams was conducted on duplicate 1-g samples of tissue from both control and ^{14}C-DDT-exposed clams before addition of the vitamin-mineral supplement. The samples were extracted in a blender with 35% water/acetonitrile and then partitioned with 100 ml of petroleum ether. Cleanup was performed as described for the adult fish.

Liquid Scintillation

Radiometric methods were used to determine DDT residues attributed to the food for all samples. Analysis was performed on a Packard Tri-Carb Liquid Scintillation Spectrometer. Samples were prepared from the portion of the extracted tissue residues not used in gas chromatographic analysis.

The samples were evaporated to 0.2 ml in glass concentrator tubes and were then transferred to glass scintillation vials with four 1-ml washings of toluene. Fifteen milliliters of Instagel‡ (scintillation cocktail) were added to each vial. The vials were kept in the scintillation counter overnight to allow them to cool and dechemiluminess before analysis.

A correlation was made between the gas chromatograms and scintillation counts through a series of dilutions of the ^{14}C-DDT stock solution. An average count per minute per microgram of DDT was then calculated and used for the determination of the micrograms of DDT attributed to the contaminated food source. Individual sample counts per minute were corrected for background radiation and sample volume removed for gas-chromatograph analysis before final calculations were made. Final results were calculated on a whole-body wet-weight basis.

STATISTICS

All survival and egg-hatchability data were transformed to arcsin $\sqrt{\%}$. Two-way analysis of variance was applied to all survival, embryo-hatchability, percentage lipid, and 30- and 60-day progeny growth data to determine the DDT effect from food or water exposure. Dunnett's procedure (Steel and Torrie, 1960) was used for comparison of treatment means with control means. Non-linear least square estimation was used to determine values for the food and water parameters that affected adult survival. Regression analysis was performed on tissue-residue data obtained from the elimination study.

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‡Instagel was purchased from Packard Instrument Company, Inc., Downers Grove, Illinois.

SECTION V

RESULTS

ADULT FISH

To simplify the presentation of results test treatments are coded as follows:

DDT Exposure	Coding
Clean water, clean food (Control)	C
Clean water, DDT food	F
0.5 µg/l DDT water, clean food	0.5 W
0.5 µg/l DDT water, DDT food	0.5 W + F
2.0 µg/l DDT water, clean food	2.0 W
2.0 µg/l DDT water, DDT food	2.0 W + F

Results from duplicate chambers were combined, and the data are expressed as the mean ± standard error (S.E.) unless otherwise indicated.

Determined DDT water concentrations in the test exposures are presented in Table 1.

Figures 1-3 show the total DDT tissue residues (DDT + DDE + TDE) at the various sample periods. An equilibrium with dietary total DDT occurred within 28-56 days (Figure 1). Figures 2-3 demonstrate the additive residue effect of dietary total DDT when compared to residues from a water source only. In general, total residues peaked by 56 days for fish exposed at F and 0.5 W and by 112 days for the rest of the exposures. An equilibrium may have been reached at 0.5 W within 56 days. In general, residue levels decreased rapidly during the spawning period (112-224 days) and then increased after termination of spawning activity. Residue levels fluctuated greatly, and apparently neither sex was affected more than the other.

TABLE 1. DDT WATER CONCENTRATIONS IN EXPOSURE CHAMBERS

Nominal DDT water concentration (µg/l)	Measured concentration (µg/l)		
	N	Mean	Range
2.0 W	41	1.53 (0.35)[a]	0.82[b]-2.30
2.0 W + F[c]	41	1.48 (0.30)	0.82[b]-2.00
0.5 W	41	0.35 (0.11)	0.16[b]-0.70
0.5 W + F[c]	41	0.37 (0.11)	0.19[b]-0.79
F[c]	41	0.01 (0.03)[d]	0.00-0.10[d]
C (Control)	41	0.00 (0.01)	0.00-0.06[e]

[a]Standard deviation in parentheses.

[b]Syringe injector malfunction (leakage).

[c]Fed clam tissue with 14C-DDT (45.6 mg/kg).

[d]Leaching from 14C-DDT food.

[e]Possible contamination from wrong food; occurred only once during exposure.

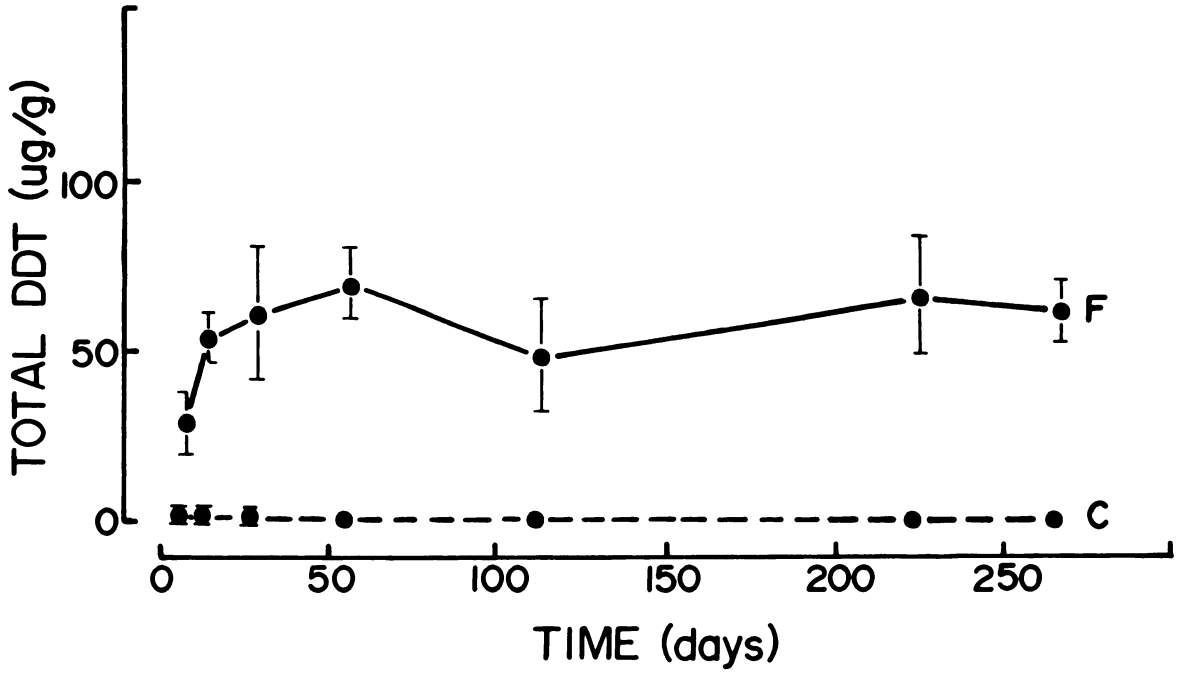

Figure 1. Total DDT residues (µg/g) in the controls and fish exposed to DDT in the food. (Vertical lines indicate standard error.)

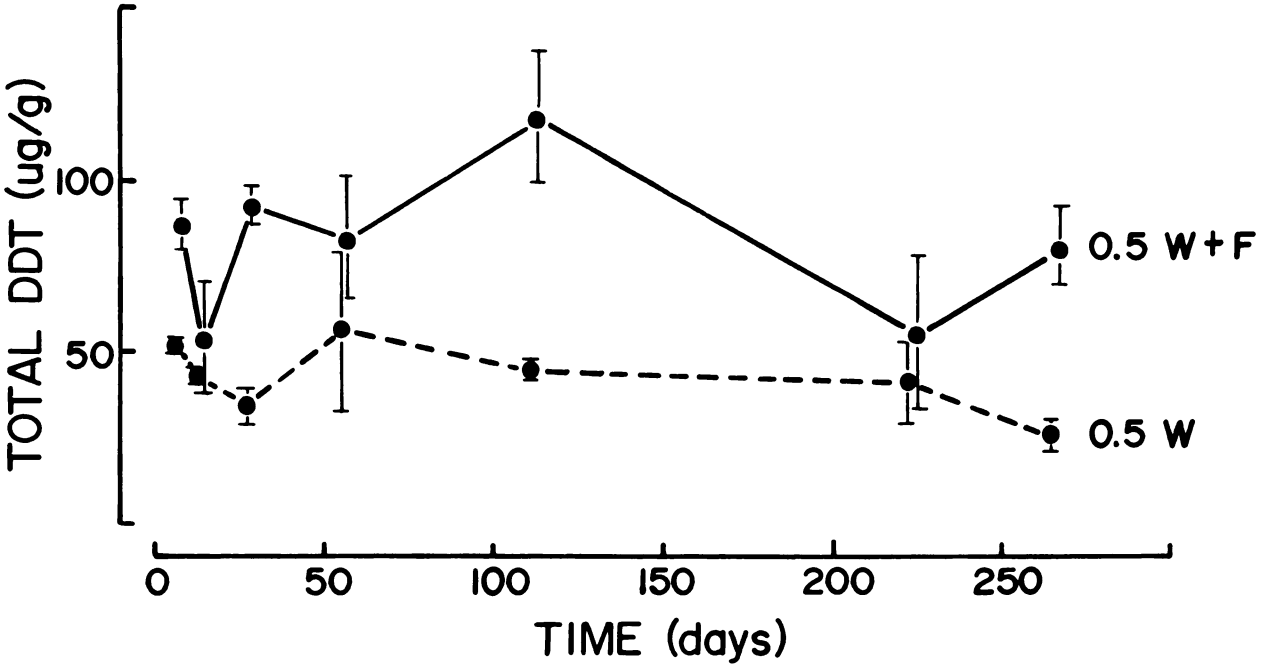

Figure 2. Total DDT residues (µg/g) in fish exposed to DDT in the water (0.5 µg/l) or in combination of food and water. (Vertical lines indicate standard error.)

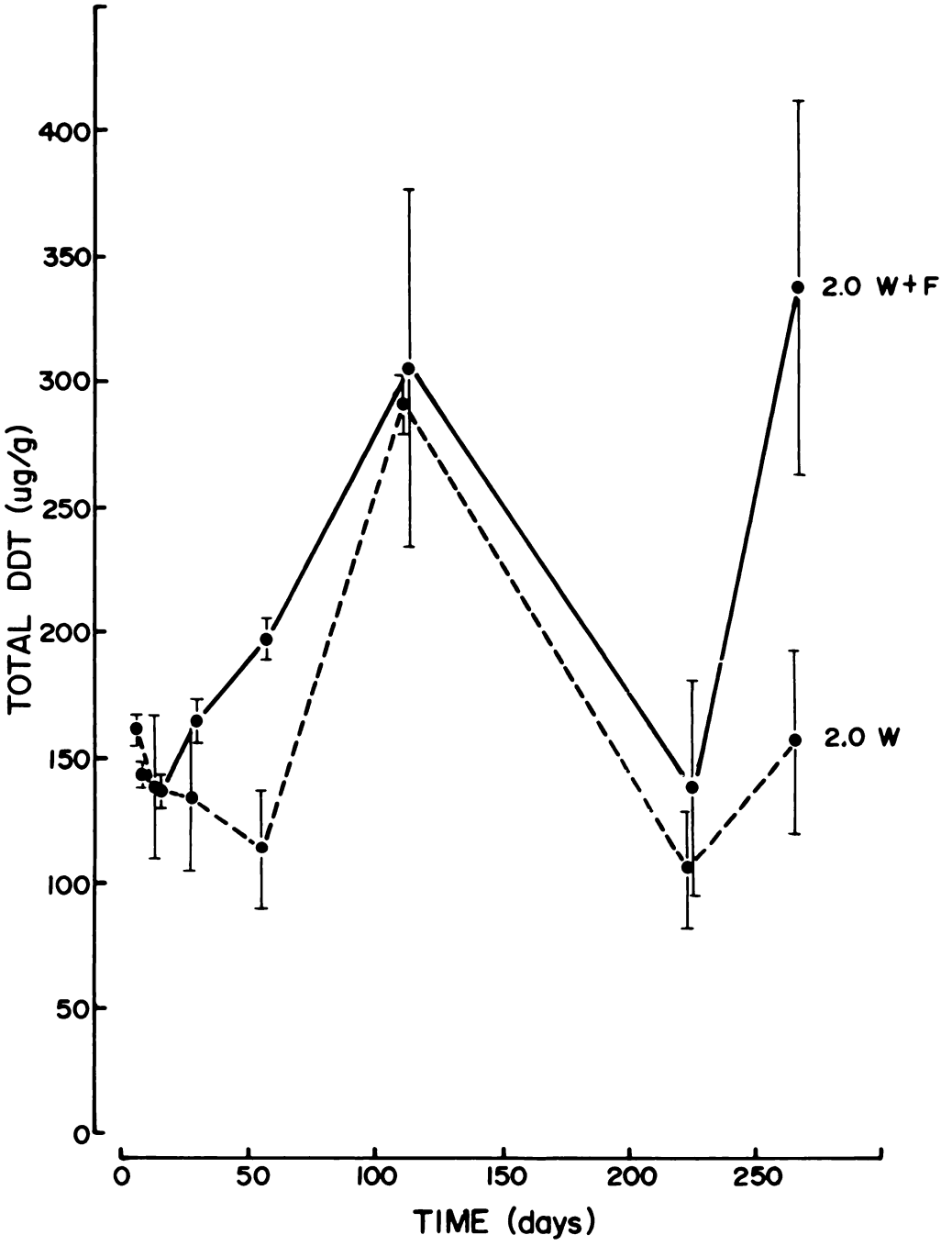

Figure 3. Total DDT residues (µg/g) in fish exposed to DDT in the water (2.0 µg/l) or in combination of food and water. (Vertical lines indicate standard error.)

Residue levels after 14 days of exposure were for the most part greater for fish exposed to DDT in both the water and diet than for those exposed in the water only. After 266 days fish from the F exposure had a mean body burden 2.4 times those exposed at 0.5 W, fish exposed at 0.5 W + F had mean residues three times those exposed at 0.5 W, and mean residues in fish exposed at 2.0 W + F were about two times greater than in fish exposed at 2.0 W.

The highest mean total DDT body burdens (µg/g) achieved for each exposure group were as follows: C, 2.0 µg/g at 14 days; F, 69 µg/g at 56 days; 0.5 W, 56 µg/g at 56 days; 0.5 W + F, 118 µg/g at 112 days; 2.0 W, 291 µg/g at 112 days; and 2.0 W + F, 337 µg/g at 266 days.

Total DDT residues are presented as DDT, DDE, and TDE in Table 2. DDE was the principal constituent found after 14 days of exposure, an indication that DDT was rapidly metabolized. Lack of TDE in the residues of fish fed DDT-exposed clam tissue until 112 days was caused by failure of the gas-chromatographic column to differentiate p,p' TDE from o,p' DDT. Column changes permitted differentiation after 56 days. In general, DDT levels decreased rapidly after 14 days. DDT, DDE, and TDE residues decreased at spawning time except at the F exposure, where DDE alone increased. TDE residues were low at 224 days for fish exposed to DDT in the food. Analysis of the DDT-exposed clam tissue is presented in Table 3. The clams metabolized very little DDT to DDE; the principal metabolite was TDE.

The 14C-DDT content of the clam meat from the four exposures averaged 45.6$\pm$3.8 µg/g (n=4) as determined by gas chromatography. This value indicates a concentration factor of 25,000 times based upon the average measured DDT water concentration. Total DDT in the clam tissue consisted of 68% DDT, 1% DDE, and 31% TDE.

Use of 14C-labeled DDT in the contaminated food allowed the separation of DDT contributed by the food by liquid-scintillation analysis (L.S.) from the total amount of DDT as determined by gas-chromatographic analysis (G.C.). Gas-chromatograph and liquid-scintillation results were not identical as they should have been for the fish exposed to 14C-DDT-contaminated food only. With n=19, the mean liquid-scintillation value

TABLE 2. DDT, DDE, AND TDE RESIDUES (µg/g) IN FISH EXPOSED TO VARIOUS TEST CONDITIONS (DUPLICATE SAMPLES COMBINED)[a]

Nominal DDT water concentration (µg/l)	Days of exposure																					
	7			14			28			56			112			224			266		
	DDT	DDE	TDE	DDT	DDE	TDE	DDT	DDE	TDE	DDT	DDE	TDE	DDT	DDE	TDE	DDT	DDE	TDE	DDT	DDE	TDE
C	1.0 (0.5)[b]	0.7 (0.2)	0.1 (0.1)	1.0 (0.2)	1.0 (0.0)	0.0	0.6 (0.1)	1.0 (0.5)	0.0	0.2 (0.0)	0.3 (0.1)	0.0	0.1 (0.0)	0.2 (0.0)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	0.0	0.0	0.1 (0.2)	0.0
F	16.2 (5.7)	12.0 (3.4)	0.0	28.2 (3.2)	24.1 (4.0)	1.0 (0.1)	23.8 (4.7)	35.3 (12.9)	1.1 (1.1)	18.5 (1.5)	49.4 (8.6)	1.0 (0.2)	7.0 (1.9)	38.5 (13.9)	15.0 (3.8)	5.2 (1.7)	47.5 (11.7)	12.5 (3.8)	5.0 (1.0)	33.3 (4.6)	22.4 (3.9)
0.5 W	26.1 (0.6)	24.9 (0.8)	0.0	18.4 (5.4)	24.3 (4.7)	0.0	7.2 (2.0)	25.5 (2.9)	0.7 (0.0)	13.2 (6.8)	18.8 (7.4)	0.6 (0.6)	6.1 (0.3)	36.8 (1.0)	3.7 (0.7)	3.5 (1.0)	36.7 (11.4)	0.8 (0.2)	2.7 (0.7)	21.7 (3.2)	1.0 (0.2)
0.5 W + F	47.4 (5.4)	39.0 (1.7)	0.0	22.5 (10.6)	29.5 (5.2)	1.4 (0.5)	35.9 (7.4)	53.7 (2.0)	3.1 (0.6)	17.7 (3.4)	63.3 (14.4)	1.4 (0.5)	14.2 (5.1)	120.2 (33.8)	20.4 (5.2)	4.9 (2.1)	41.7 (17.2)	7.9 (3.7)	7.5 (1.5)	54.1 (7.0)	18.2 (2.9)
2.0 W	94.2 (5.3)	66.8 (0.9)	0.0	48.5 (8.8)	88.3 (19.6)	1.4 (0.3)	37.0 (13.9)	92.0 (14.1)	4.0 (0.9)	25.1 (8.2)	86.6 (14.8)	1.7 (0.3)	58.0 (0.6)	230.9 (34.9)	19.3 (1.6)	9.5 (2.5)	93.6 (20.7)	9.1 (1.0)	15.8 (4.6)	136.9 (31.4)	3.8 (0.8)
2.0 W + F	92.8 (4.9)	50.3 (0.0)	0.0	77.8 (23.2)	54.7 (28 3)	3.8 (0.8)	43.3 (3.7)	113.4 (12.1)	7.4 (0.2)	44.6 (0.5)	149.2 (8.4)	3.1 (0.9)	49.8 (19.0)	246.6 (67.8)	52.2 (13.5)	18.0 (7.3)	113.3 (32.8)	6.2 (3.3)	28.5 (8.1)	291.7 (62.0)	17.1 (5.1)

[a]Each sample is a composite of 5-10 fish.

[b]()Standard error.

TABLE 3. DDT, DDE, AND TDE RESIDUES (μg/g) IN THE FISH FOOD

Exposures	Time period when used as food (days)	DDT	DDE	TDE	Time of year collected	Time of year exposed to 14C-DDT
		14C-DDT-exposed clams				
1	0-112	42.6[a]	0.6	14.3	Fall	Spring (late)
2	112-224	38.2	0.5	2.7	Spring	Fall
3	112-224	31.2	0.2	9.7	Fall	Winter
4	224-266	11.0	0.2	29.0	Fall	Spring (early)
		Clean clams				
1	0-112	0.7	-[b]	-	Fall	Spring (late)
2	112-224	-	-	-	Spring	Fall
3	112-224	-	-	-	Fall	Winter
4	224-266	-	-	-	Fall	Spring (early)

[a]Ovendried weight (1 1/2-2 hr; 110° C).

[b]Not detectable.

was 110% of the gas-chromatograph value with a standard deviation of 28.4%. To correct for this difference L.S. values were adjusted to G.C. values by the following formula: adjusted L.S. value = L.S. value obtained/ratio L.S. value to G.C. value for F exposed fish. These data are presented in Table 4 and are expressed as a percentage of the total residues as determined by gas-chromatographic analysis. It appears that DDT in the food had a maximum input within 28-56 days. The relative amount contributed by the DDT-contaminated food was about 60% of the total DDT residues in fish exposed at 0.5 W + F and about 30% in fish exposed at 2.0 W + F.

Mean calculated accumulation of total DDT from food and water was 1.2 $\pm$ 0.1 times for the DDT from the food (n=57), 99,000$\pm$7,000 times for DDT from the water (n=39, 0.5 W and 2.0 W exposures combined), and 87,000$\pm$9,000 times from the water (n=38, 0.5 W + F and 2.0 W + F exposures combined) after the food contribution was substracted. If all water-exposure samples were combined, a mean accumulation (n=77) of 93,000$\pm$6,000 times was obtained.

Lipid percentages were also determined for the test fish. Conversion of lipid values to $\arcsin\sqrt{\text{percent lipid}}$ and analysis by two-way analysis of variance indicated that there was no significant difference (P=0.05) in percentage of lipids between fish exposed to DDT in the water only and fish exposed to DDT in the water and fed DDT-contaminated food.

Mortality results were analyzed by calculation of the accumulative probability of fish survival over the different sample periods during the toxicity test. This was accomplished by obtaining an estimate of the probability of a fish surviving from time tj to tj_{+1} given that it was alive at time tj and exposure started at time t_o=0. This estimate is (1) $Pj_{+1}=Nj_{+1}/nj$, where nj is the number of fish alive and exposed at time tj; it assumes that no fish were removed during the interval. Since samples were taken at the end of each interval, the probability of survival for each interval had to be computed separately, and the probability (Ps) of survival for the entire period is (2) $Ps=\prod_{j=1}^{m} Pj$, where m is the number of sampling points. In Table 5 the computed probability of survival for each exposure is shown where m=7. To test whether the addition of DDT-contaminated food altered the probability of survival, a two-way analysis of variance was run on the data

TABLE 4. PERCENTAGE OF TOTAL DDT CAUSED BY THE 14C-DDT FOOD SOURCE (DUPLICATE SAMPLES COMBINED)

Nominal DDT water concentration (µg/l)	Days of exposure							Mean for entire period
	7 (n=2)	14 (n=2)	28 (n=2)	56 (n=2)	112 (n=2)	244 (n=4)	266 (n=6)	
F	100.0[a](1.4)[b]	100.0[c]	100.0 (2.8)	100.0 (13.4)	100.0 (6.2)	100.0 (2.7)	100.0 (3.0)	100.0 (1.6) (n=19)
0.5 W + F	22.5 (0.0)	46.9 (19.4)	76.4 (3.4)	81.7 (4.2)	55.4 (1.4)	72.2 (5.4)	64.7 (1.4)	62.1 (4.2) (n=20)
2.0 W + F	4.8 (2.1)	28.1 (15.7)	38.2 (3.4)	50.7 (5.6)	33.9 (0.9)	28.7 (2.3)	17.6[d](1.2)	27.6 (3.4) (n=18)

[a]Liquid-scintillation values shown are adjusted.

[b]( ) Standard error.

[c]n=1.

[d]n=4.

TABLE 5. ESTIMATED PROBABILITY OF SURVIVAL FOR FISH EXPOSED TO VARIOUS TEST CONDITIONS FOR 266 DAYS

		Nominal DDT water concentration (µg/l)		
		0	0.5	2.0
Clean food	Tank A	0.8700	0.7993	0.5234
	Tank B	0.9052	0.8697	0.4211
14C-DDT contaminated food	Tank A	0.6248	0.7369	0.1837
	Tank B	0.8860	0.7392	0.2541

after arcsin $\sqrt{Ps}$ transformation was employed. The hypothesis that the presence of DDT in the food does not change survival was rejected at the P=0.025 level.

The accumulative reduction in the probability of survival is presented in Figure 4. Two definite periods of high mortality are indicated, the juvenile stage during the first 28 days of exposure (test started with about 45-day-old fish; therefore, the fish were 73 days old at 28 days' exposure) and at spawning time between 112 and 224 days of exposure. Mortality was greater in fish fed DDT-contaminated food and it remained high into 56 days' exposure before a plateau was reached, whereas the death rate among fish exposed at a corresponding DDT water concentration, but fed clean food, reached a plateau at 28 days. The death rate was also greater for fish fed DDT-contaminated food during the spawning period. Fish that died during the spawning period were predominantly highly colored adult males (about 83% males, 17% females). Residue levels in the dead fish were only about 55% of those in live sampled fish. Lipid percentages, however, were very low (mean 1.07% (n=23), all samples combined) when compared to the live fish at the corresponding time period (mean 3.1% (n=12), all samples combined). The residue levels attributed to DDT in the food among fish that died was all the DDT at the F exposure, 70% at the 0.5 W + F exposure, and 23% at the 2.0 W + F exposure. These percentages are similar to those found in the similarily exposed live fish.

EMBRYOS

Embryo-hatchability data are presented in Table 6. Data were transformed to arcsin $\sqrt{\text{percent hatch}}$ and analyzed by two-way analysis of variance. Presence of DDT in the parents' food did not significantly alter hatchability, whereas DDT in the water did (P=0.05). Hatchability reduction, however, was significant only for embryos from parent fish that were subjected to the 2.0 W exposure. Table 7 shows the total DDT residues at the various exposures and also the percentage DDT from food for embryos from adult fish fed the DDT-contaminated food. The percentages observed are similar to those for the adult fish. Presence of DDT-contaminated food appears to be additive. Addition of the residues found in embryos from fish at the F exposure to residues found in embryos from fish at the 0.5 W and 2.0 W exposures will

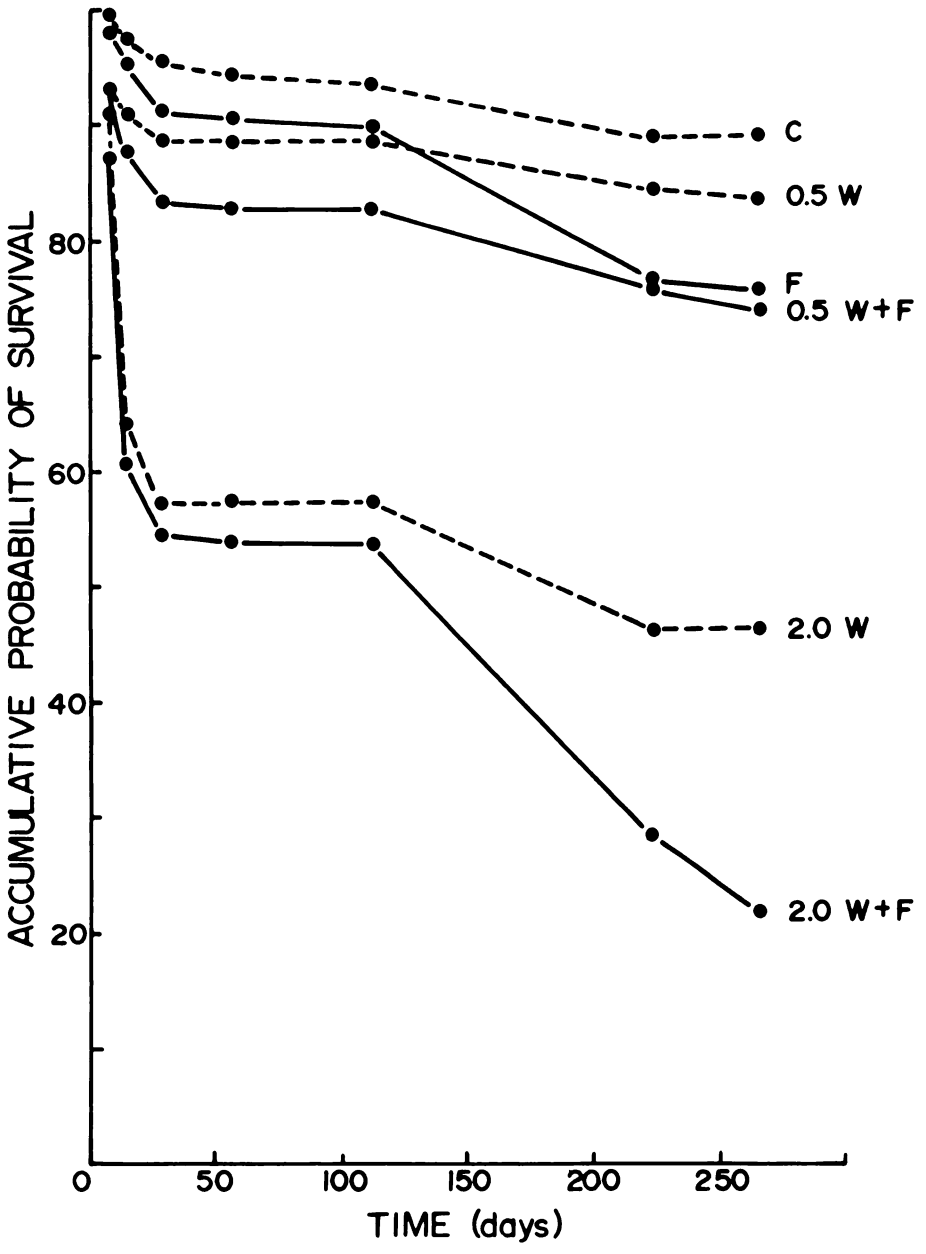

Figure 4. Accumulative probability of survival for fish that had been exposed to DDT.

TABLE 6. HATCHABILITY OF EMBRYOS AT VARIOUS TEST EXPOSURES

(DUPLICATE SAMPLES COMBINED)

Nominal DDT water concentration (µg/l)	N[a]	Number of eggs set up	Number of eggs hatched	Percentage hatch	Range (percentage hatch)	Arcsin $\sqrt{\text{percentage hatch}}$
C	62	3,473	3,089	88.9	54-100	70.54
F	44	2,610	2,241	85.7	38-100	67.78
0.5 W	51	2,550	2,152	84.4	46-100	66.74
0.5 W + F	55	3,491	3,114	89.2	48-100	70.81
2.0 W	98	6,759	5,009	74.1[b]	12-100	59.41
2.0 W + F	51	3,117	2,440	78.3	44-100	62.24

[a] N=number of spawnings.

[b] Significantly different from the control ($\alpha 0.05$) Dunnett's procedure (Steel and Torrie, 1960).

TABLE 7. TOTAL DDT RESIDUES (µg/g) IN EMBRYOS FROM FISH EXPOSED TO VARIOUS TEST CONDITIONS, AND PERCENTAGE TOTAL DDT CONTRIBUTED BY THE CONTAMINATED FOOD (DUPLICATE SAMPLES COMBINED)

Nominal DDT water concentration (µg/l)	Number of samples	Mean (µg/g)	Range (µg/g)	Percentage due to DDT in the food: Liquid scintillation adjusted
C	15	0.4 (0.0)[a]	0.1-0.6	-
F	12	12.0 (1.1)	6.4-18.8	100 (6.2)
0.5 W	16	6.7 (0.7)	3.3-11.9	-
0.5 W + F	15	18.9 (3.1)	5.4-43.8	68 (1.8)
2.0 W	23	24.0 (2.0)	12.2-50.4	-
2.0 W + F	18	40.9 (3.8)	21.9-78.0	28 (0.5)

[a] ( ) Standard error.

give residue levels close to those for embryos from fish at the 0.5 W + F and 2.0 W + F exposures. Residues in the embryos from fish exposed to DDT in the food and in the water are two times greater than in those from fish exposed to DDT only in the water.

To determine the relative amount of DDT that might be transferred to the embryo from the fish, control embryos were placed in the 2.0 µg/l DDT water exposure for 24 hr (embryos would not have been exposed to DDT in the water any longer than this before collection for residue analysis). These embryos had a residue level of 0.95 µg/g, which would probably be the maximum that embryos spawned in this tank could have attained from the water. Residue levels found in the latter embryos were much higher; therefore residues found were mostly transferred from the adult fish.

Separation of total DDT to DDT, DDE, and TDE is shown in Table 8. DDE was the primary constituent. Some DDT from the water was metabolized to TDE, but the amount is only about 10% of that attributed to the food source. There is increased TDE with higher water exposure for the embryos from the water plus food-exposed fish. Mean accumulation of DDT was 0.26±0.02 times (n=45) from the food source for embryos from parent fish at the F exposure, 17,000±1,000 times (n=39) from the water for those from parent fish exposed at 0.5 W and 2.0 W, and 19,000±2,000 times (n=33) for those from fish exposed in combination, to 0.5 W + F and 2.0 W + F after the residues due to food were subtracted.

LARVAE AT HATCH

Total DDT residue data and percentages of residues caused by DDT-contaminated food are presented in Table 9. When compared with embryo residues, residues in larvae at hatch from parent fish fed dietary DDT are about two times higher, whereas those from parent fish exposed to DDT only in the water are about 3.6 times higher. Part of this difference is explained by the reduction in weight of the larvae at hatch to almost one-half that of the embryos. Therefore, if nearly all the residue were contained within the developing larva, the calculated residue level would automatically be two times greater at hatch when the embryo membrane and surrounding fluid is lost. The percentage of total DDT resulting from DDT-contaminated food was about 8% lower for larvae at hatch than for embryos. Larvae were removed for analysis daily, so it is possible that some may have been exposed to DDT in the water for a maximum of 24 hr before being removed.

Separation of total DDT to DDT, DDE, and TDE is shown in Table 10. DDE again is the principal constituent. All residues are greater than those observed for embryos. High TDE levels in larvae from food-exposed parents are attributed to the food source. Mean accumulation of DDT in larvae from fish exposed to the two DDT sources was 0.53±0.03 times (n=16) for dietary DDT (double that for embryos), 62,000±4,000 times (n=13) for DDT water exposure alone, and 50,000±5,000 times (n=11) for exposure to DDT in the water minus that contributed by the food.

TABLE 8. DDT, DDE, AND TDE RESIDUES (µg/g) IN EMBRYOS FROM FISH EXPOSED TO VARIOUS TEST CONDITIONS (DUPLICATE SAMPLES COMBINED)

Nominal DDT water concentration (µg/l)	DDT	DDE	TDE
C	0.08 (0.0)[a]	0.35 (0.0)	-[b]
F	1.56 (0.1)	7.76 (0.9)	2.75 (0.2)
0.5 W	0.75 (0.1)	5.70 (0.6)	0.30 (0.0)
0.5 W + F	1.99 (0.4)	13.66 (2.2)	3.26 (0.6)
2.0 W	3.83 (0.5)	19.61 (1.7)	0.58 (0.1)
2.0 W + F	5.31 (0.4)	31.32 (2.9)	4.24 (0.6)

[a]( ) Standard error.

[b]Not detectable.

TABLE 9. TOTAL DDT RESIDUES (µg/g) IN LARVAE AT HATCH FROM FISH EXPOSED TO VARIOUS TEST CONDITIONS, AND PERCENTAGE TOTAL DDT CONTRIBUTED BY THE CONTAMINATED FOOD (DUPLICATE SAMPLES COMBINED)

Nominal DDT water concentration (µg/l)	Number of samples	Mean	Range	Percentage due to food
				Liquid scintillation adjusted
C	6	0.17 (0.01)[a]	0.11-0.19	-
F	4	26.4 (2.9)	19.1-31.5	100 (0.8)
0.5 W	4	24.0 (2.2)	17.8-27.2	-
0.5 W + F	6	43.5 (4.3)	33.5-62.4	60 (1.9)
2.0 W	9	87.9 (5.9)	54.0-113.2	-
2.0 W + F	5	96.8 (17.8)	65.4-166.5	20 (0.6)

[a]( ) Standard error.

TABLE 10. DDT, DDE, AND TDE RESIDUES (µg/g) IN LARVAE AT HATCH FROM FISH EXPOSED TO VARIOUS TEST CONDITIONS (DUPLICATE SAMPLES COMBINED)

Nominal DDT water concentration (µg/l)	DDT	DDE	TDE
C	-[a]	0.17 (0.0)[b]	-[a]
F	2.96 (0.3)	18.63 (2.6)	4.83 (0.2)
0.5 W	3.77 (0.3)	18.89 (2.3)	1.32 (0.0)
0.5 W + F	5.70 (0.5)	32.77 (4.5)	4.99 (0.6)
2.0 W	19.85 (0.9)	66.49 (5.3)	1.53 (0.1)
2.0 W + F	20.11 (2.0)	72.99 (15.4)	2.89 (0.7)

[a]Not detectable.

[b]( ) Standard error.

PROGENY AT 30 AND 60 DAYS

Residue data and tissue-residue percentages caused by DDT-contaminated food are shown in Table 11. The additive DDT food effect is again indicated. Residues are slightly higher at 60 than at 30 days, except for fish at the F exposure, where they are lower. The percentage of total DDT caused by the food source is only 8% higher at 60 days than at 30 days. Progeny from embryos from fish exposed to 2.0 W hatched and raised in control water for 30 days contained only 0.5 µg/g total DDT in their tissues. Progeny from embryos from fish exposed to 2.0 W + F, also hatched and raised in control water and fed clean food for 30 days, had residues of only 0.2 µg/g total DDT. As determined by liquid-scintillation analysis, none of this DDT could be traced to the DDT-contaminated food intake by parent fish. Progeny, however, from embryos from the same parent fish that were hatched and raised in clean water but fed DDT contaminated food for 30 days had total DDT residues of 31.6 µg/g, of which 93% could be attributed to the food.

Residues in 60-day progeny are not much different from residues in the corresponding parent fish at 14 days' exposure (fish were ~59 days old) if calculated on a percentage lipid basis. These values are as follows: 60-day progeny fed DDT food, 8.41 µg/g, parent fish at 59 days old, 14.4 µg/g; 0.5 W 60 days, 7.1 µg/g, parent fish 59 days old, 10.7 µg/g; and 0.5 W + F 60 days, 17.9 µg/g, parent fish, 59 days old, 21.8 µg/g.

Separation of total DDT residues to DDT, DDE, and TDE are presented in Table 12. In general, DDT residues decreased at most exposures between 30 and 60 days, whereas DDE residues increased. TDE residues also showed a slight increase between 30 and 60 days, except at the 0.5 W + F exposure where there was a 38% increase.

Mean accumulation of total DDT from the food was 0.70±0.13 times (n=8) for 30-day progeny and 0.75±0.09 times (n=4) for 60-day progeny. Total DDT in the water was accumulated 39,000±5,000 times (n=4) for 30-day and 70,000±12,000 times (n=5) for 60-day progeny exposed to DDT only in the water, whereas DDT in the water was magnified 70,000±

TABLE 11. TOTAL DDT (µg/g) IN 30- AND 60-DAY-OLD PROGENY OF FISH EXPOSED TO VARIOUS TEST CONDITIONS, AND PERCENTAGE OF TOTAL DDT CONTRIBUTED BY THE CONTAMINATED FOOD (DUPLICATE SAMPLES COMBINED)

(a) 30-Day-old progeny

Parent fish nominal DDT water concentration (µg/l)	Number of samples	Mean	Range	Percentage caused by DDT in the food: Liquid scintillation adjusted
C	3	0.20 (0.0)[a]	0.16-0.23	-
F[b]	4	35.70 (1.6)	31.50-39.10	100.0 (9.4)
0.5 W	4	13.70 (1.8)	10.00-17.90	-
0.5 W + F[b]	4	46.4 (4.9)	37.20-60.20	60.0 (16.3)
2.0 W	3	0.5 (0.2)	0.33-0.93	-
2.0 W + F[c]	1	0.2	-	0.0
2.0 W + F[d]	1	31.6	-	93.0

[a] ( ) Standard error.

[b] Progeny fed DDT-contaminated food.

[c] Progeny hatched and raised in control water and fed clean food.

[d] Progeny hatched and raised in control water and fed DDT food.

(b) 60-Day-old progeny

Parent fish nominal DDT water concentration (µg/l)	Number of samples	Mean	Range	Percentage caused by DDT in the food: Liquid scintillation adjusted
C	3	0.21 (0.0)[a]	0.15-0.30	-
F[b]	2	28.60 (5.7)	22.9-34.2	100 (4.1)
0.5 W	5	24.00 (4.0)	14.3-38.1	-
0.5 W + F[b]	2	58.20 (2.4)	55.8-60.6	68 (0.4)

[a] ( ) Standard error.

[b] Progeny fed DDT-contaminated food.

TABLE 12. DDT, DDE, AND TDE RESIDUES (µg/g) IN 30- AND 60-DAY-OLD PROGENY OF FISH EXPOSED TO VARIOUS TEST CONDITIONS (DUPLICATE SAMPLES COMBINED) (LARVAE WERE EXPOSED TO SAME DDT EXPOSURE AS PARENT FISH)

Nominal DDT water concentration (µg/l)	Progeny exposure (days)	DDT	DDE	TDE
C	30	0.08 (0.01)[a]	0.08 (0.01)	0.04 (0.04)
	60	0.08 (0.01)	0.20 (0.04)	-
F	30	8.07 (2.00)	15.38 (2.90)	12.21 (3.20)
	60	4.72 (0.40)	11.73 (0.70)	12.33 (5.80)
0.5 W	30	4.64 (0.90)	8.54 (0.90)	0.51 (0.20)
	60	6.08 (0.40)	17.34 (3.50)	0.55 (0.20)
0.5 W + F	30	12.51 (3.30)	18.60 (3.10)	15.27 (2.40)
	60	10.34 (1.00)	26.83 (0.50)	21.08 (0.90)

[a]( ) Standard error.

2,000 times (n=3) for 30-day and 51,000±4,000 times (n=2) for 60-day progeny exposed to DDT in the water after the food contributed portion was substracted.

DDT apparently did not affect the growth of the 30- and 60-day progeny. Two-way analysis of variance on growth data indicated that there was no significant growth difference (P=0.05) between the progeny exposed to DDT in the food or water.

All larvae died within 5 days of hatch at the 2.0 W or 2.0 W + F exposures. Control larvae, in groups of 40 each (n=4), transferred to these concentrations also died within 5 days. Survival data (Table 13) were transformed to arcsin $\sqrt{\text{percent survival}}$ and analyzed by two-way analysis of variance. A significant effect (P=0.05) on survival was observed for DDT water exposure at both 30 and 60 days, but not for food exposure. Use of Dunnett's procedure (Steel and Torrie, 1960) indicated that the exposures significantly different from the controls were those at 2.0 W and 2.0 W + F and those from parent fish exposed to 2.0 W + F, but with the progeny hatched and raised in control water and fed clean food for 30 days. This latter group experienced a twofold higher death rate than progeny from parent fish exposed at 2.0 W that were hatched and raised in control water and fed clean food. In this higher mortality group, progeny in two groups (40 fish each) experienced zero survival and one group had 75% survival. Total DDT residue in the survivors was 0.18 µg/g. Another group of progeny from the same parent fish similarily hatched and raised in control water but fed DDT-contaminated food had only 19.5% survival with a residue level in survivors of 31.6 µg/g total DDT. Ninety-three percent of this residue level could be directly attributed to their DDT-contaminated food intake.

ELIMINATION STUDY

Elimination of total DDT from fathead minnows exposed to 0.5 W and 0.5 W + F is shown in Figure 5. Relatively little elimination occurred in fish exposed to 0.5 W, whereas a definite elimination (significant at the 0.10 level) occurred in fish exposed at 0.5 W + F. In these fish about 60% of the mean total micrograms of total DDT was lost within 56 days.

TABLE 13. PERCENTAGE SURVIVAL OF 30- AND 60-DAY OLD PROGENY FROM PARENT FISH EXPOSED TO VARIOUS TEST CONDITIONS (LARVAE EXPOSED TO SAME DDT EXPOSURE AS PARENT FISH) (A) 30-DAY SURVIVAL (B) 60-DAY SURVIVAL

(A)		Nominal DDT water concentration (µg/l) 0	0.5	2.0[a]	2.0
Clean food	Tank a	73.8 (n=4)	55.0 (n=4)	25.0 (n=2)	0 (n=3)
	Tank b	55.8 (n=3)	65.6 (n=4)	62.3 (n=2)	0 (n=3)
	Grand mean	66.1	60.3	41.0	0[b]
DDT-contaminated food	Tank a	73.0 (n=3)	57.5 (n=3)	23.6 (n=3)	0 (n=2)
	Tank b	69.9 (n=3)	36.7 (n=3)	0 (n=1)	0 (n=2)
	Grand mean	71.2	47.1	18.8[b]	0[b]

F values 3.8 df = 4.07 F cal DDT water = 23.83
1.8 df = 5.32 F cal DDT food = 2.26

[a]Progeny from parent fish were hatched and raised in clean water and fed clean food.

[b]Values significantly different from the control larvae (duplicate chambers combined), two-way analysis of variance and Dunnett's procedure (Steel and Torrie, 1960) n=8; tα = 0.05 Dunnett's - 7,8 df 30 days.
5,6 df 60 days

(B)		Nominal DDT water concentration (µg/l) 0	0.5	2.0
Clean food	Tank a	46.3 (n=2)	31.2 (n=2)	0
	Tank b	45.0 (n=1)	66.3 (n=2)	0
	Grand mean	45.8	49.0	0
DDT-contaminated food	Tank a	62.5 (n=1)	35.0 (n=1)	0
	Tank b	51.3 (n=1)	42.5 (n=1)	0
	Grand mean	57.0	38.8	0

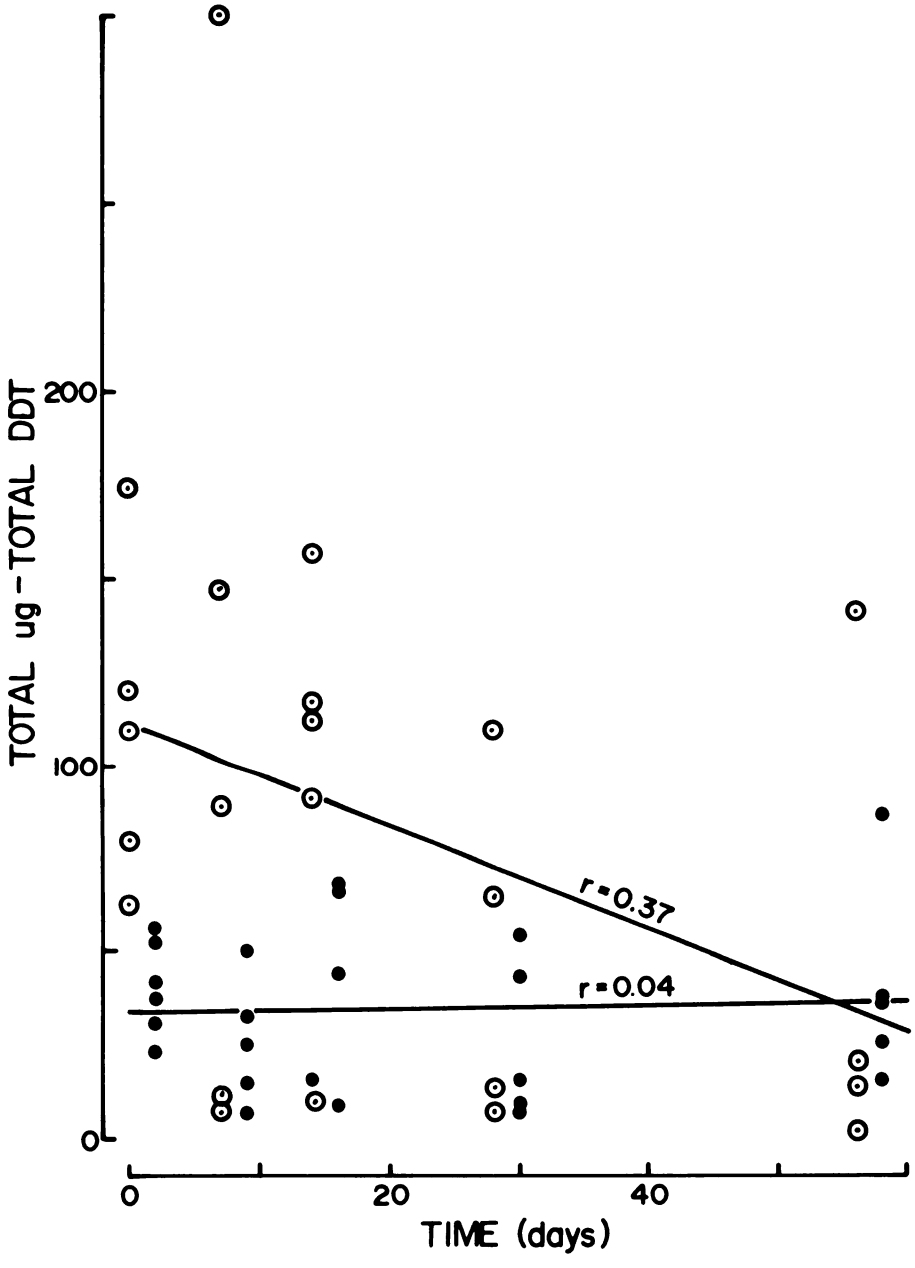

Figure 5. Elimination of total DDT from fish exposed at 0.5 W (●) and 0.5 W + F (⊙). Each point represents one fish.

Liquid-scintillation analysis of the fish exposed to DDT-contaminated food is shown in Table 14. Residues caused by food and water are expressed as a percentage of the total DDT. With longer elimination time significantly less total DDT remains that is attributed to the food, and the water contribution becomes correspondingly greater.

Separation of total DDT to DDT, DDE, and TDE and subsequent regression analysis of elimination data are presented in Figures 6-8. A significant reduction in DDT and TDE occurred for fish at 0.5 W + F, but not for those at 0.5 W ($P=0.05$). In the latter group DDT was metabolized slightly and TDE levels remained unchanged. Essentially no DDE was eliminated in the 0.5 W-exposed fish, whereas elimination did take place in fish exposed at 0.5 W + F. Comparison of the TDE residues between the two groups of fish indicates that almost all the TDE was from the food source.

TABLE 14. MEAN PERCENTAGE OF TOTAL DDT RESIDUES REMAINING IN FISH (EXPOSED TO A COMBINATION OF DDT IN FOOD AND WATER) THAT WAS ATTRIBUTED TO THE FOOD OR TO THE WATER

	Days after placed in control water				
	0	7	14	28	56
Percentage due to DDT food uptake					
Liquid scintillation adjusted	n=6 64.7 (1.4)[a]	n=5 68.7 (4.7)	n=5 74.2 (7.0)	n=5 47.5[b] (3.1)	n=4 46.0[b] (1.5)
Percentage due to DDT water uptake					
100-Liquid scintillation adjusted	35.3	31.3	25.8	52.5	54.0

[a] ( ) Standard error.

[b] Significantly different from 0 days (ANOVA and Dunnett's procedure; P=0.05; F=2.9; F cal=9.1).

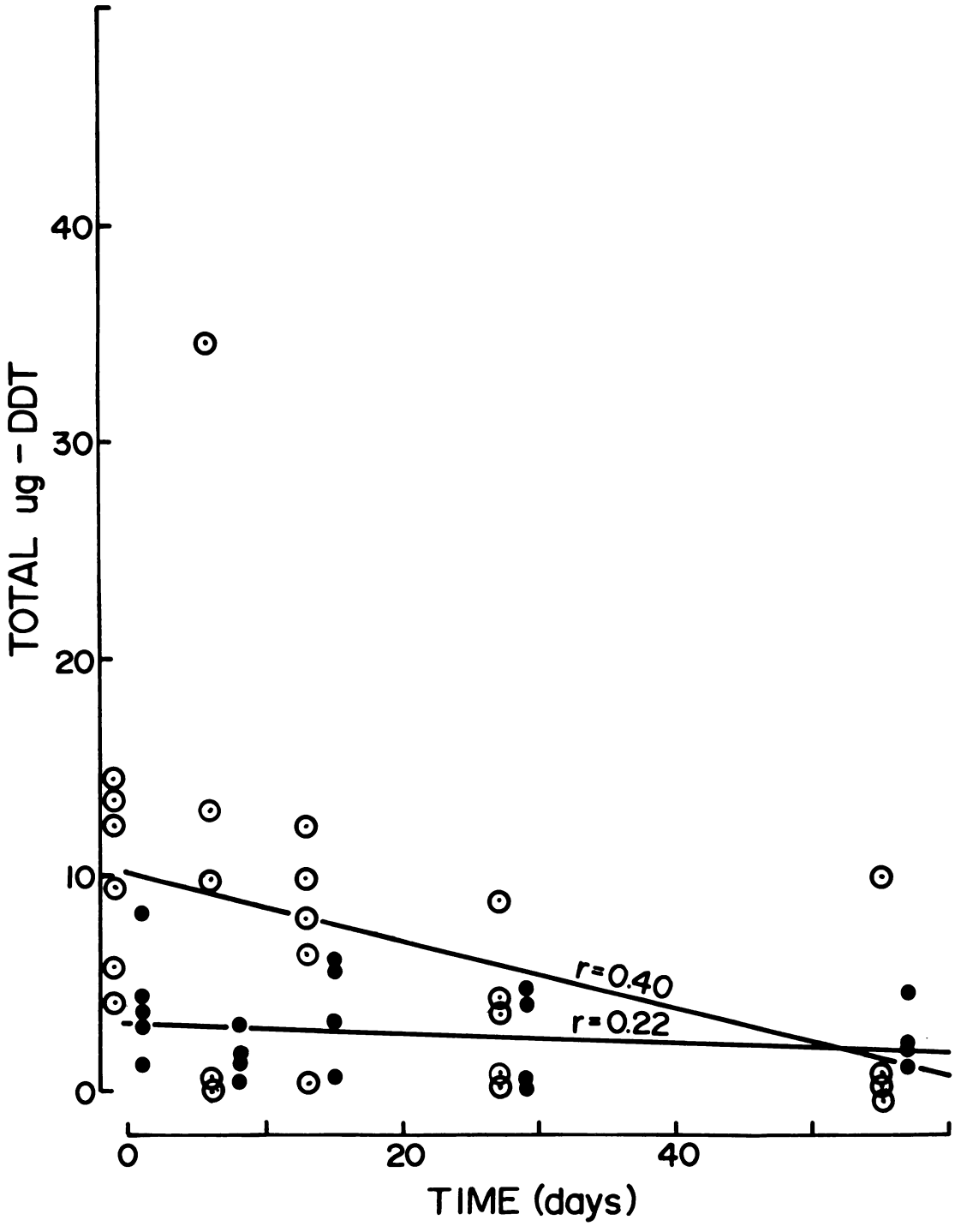

Figure 6. Elimination of DDT from fish exposed at 0.5 W (●) and 0.5 W + F (◎). Each point represents one fish.

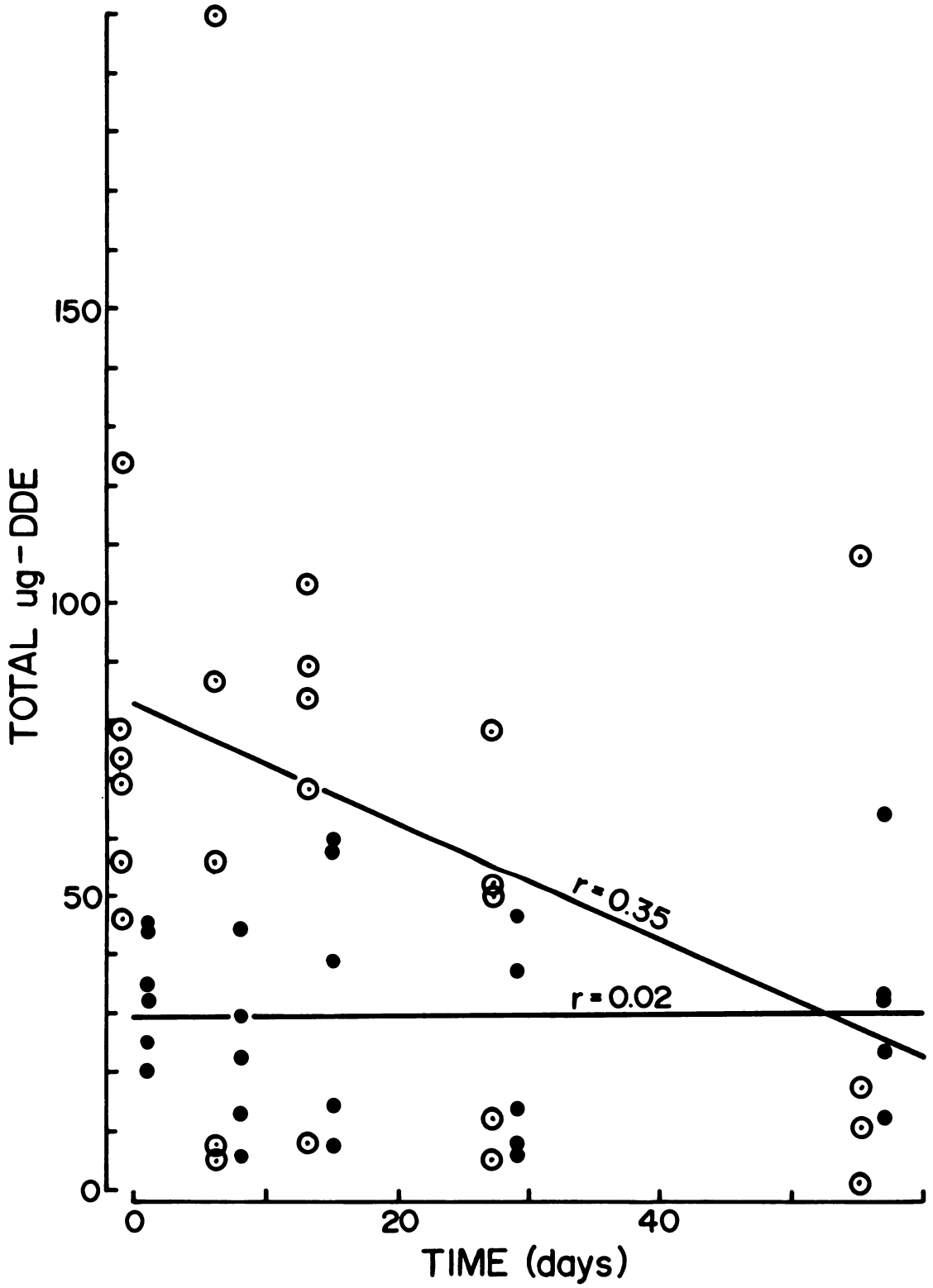

Figure 7. Elimination of DDE from fish exposed at 0.5 W (●) and 0.5 W + F (⊙). Each point represents one fish.

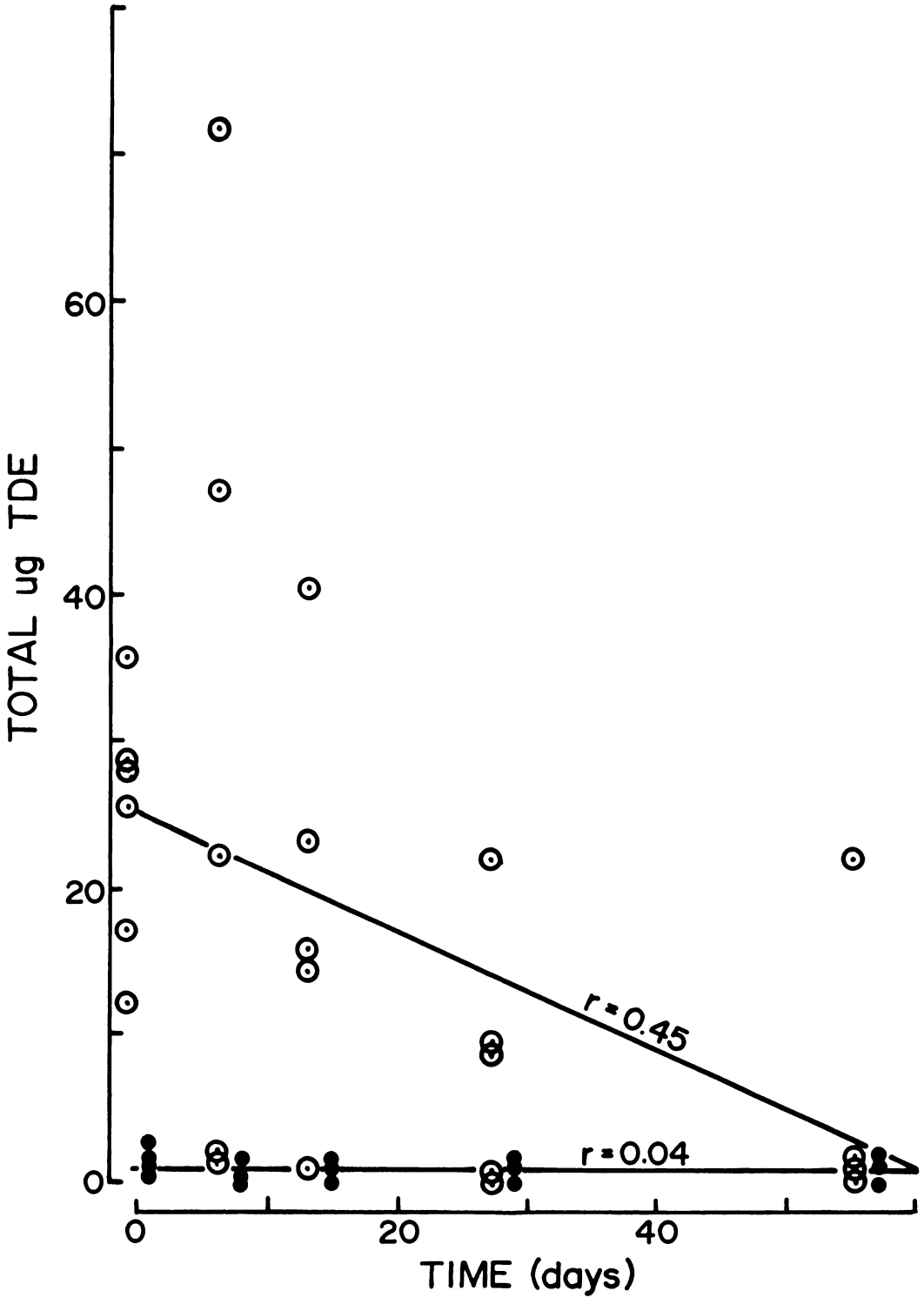

Figure 8. Elimination of TDE from fish exposed at 0.5 W (●) and 0.5 W + F (◎). Each point represents one fish.

SECTION VI

DISCUSSION

The fathead minnows did not achieve an equilibrium with DDT from the water at 2.0 µg/l, whereas they apparently did achieve equilibrium at 0.5 µg/l. Lack of equilibrium was also reported by Hamelink et al. (1971) for young-of-the-year largemouth bass exposed to DDT at 50 µg/l and greater for up to 80 days. However, this does not necessarily mean that the fish did not approach an equilibrium with the toxicant at any instant in time. During long-term studies factors such as lipid content, toxicant, stress, etc. can all be expected to influence residue concentrations. Therefore the fish probably did achieve equilibrium with the DDT in the water at various times during the test. Greater residue fluctuations at the higher DDT exposure in our test may have occurred because the fish were closer to their maximum accumulative capabilities, and any change in body conditions would be more directly reflected in residue levels. Our results indicate that uptake from the food is additive to the amount taken up from the water and that equilibrium with the food was reached within 56 days. These findings are similar to those observed by other authors. Grzenda et al. (1970) reported that there was no additional increase in body concentration in goldfish after dietary DDT exposure (18 µg/g) for 32 days. Macek et al. (1970) reported an equilibrium with dietary DDT in the liver, brain, and skeletal muscle of rainbow trout after 28 days' exposure. The portion of the total DDT tissue residues in our test that could be directly attributed to the DDT-contaminated diet was about 30% in fish exposed to a DDT water concentration equal to that at which the food had been exposed and 60% in fish exposed to a water concentration one-fourth that level. This appears to indicate that the main source of DDT uptake is the water. Chadwick and Brocksen (1969) exposed freshwater sculpins to dieldrin in diet and water and observed that a maximum of 16% of the dieldrin was accumulated from the food. They also stated that accumulation from the diet might be expected to be additive, but this was not so in their

test. They reported residue levels that were not much different between fish exposed in food and water and in water only, whereas in our test tissue residues between the two exposures were different after 14 days. Reinert (1967), also in a study with dieldrin, observed that only about one-tenth as much of the residues in guppies were accumulated through ingestion of contaminated food when compared to residues in guppies exposed to a water concentration equal to that in which the food was exposed. This test, however, was of a shorter duration (32 days). In our test the highest residue level caused by DDT-contaminated food was one-fourth that caused by DDT water exposure at the same concentration to which the food was exposed.

Our mean DDT concentration factor was about 1.2 times for dietary DDT and about 100,000 times for DDT from the water. These values are similar to those observed by other authors. Hunt and Bischoff (1960) observed a 125,000 times concentration factor for brown bullheads from water, and Courtney and Reed (1972) observed a concentration in the tissues of golden shiners exposed to DDT at 0.3 µg/l in the water of about 100,000 times after 15 days. Reinert (1967) observed a 0.05 times food concentration factor in tissue residues of guppies exposed to a diet of dieldrin-contaminated Daphnia (31 µg/g) for 32 days, but a concentration factor of 1.3 times for Daphnia fed dieldrin-contaminated algae (71 µg/g). He stated, however, that the latter concentration factor may have been caused by algae ingested but not yet assimilated, as alga cells were observed in the Daphnia digestive tract. Epifanio (1973) observed a concentration factor of 1.7 times when he fed dieldrin-contaminated Artemia salina brine shrimp nauplii (0.213 µg/g) to crab larvae. Macek and Korn (1970) observed a food concentration factor of 0.6 times for brook trout exposed to 3 µg/g DDT in their diet for 120 days. Grzenda et al. (1970) estimated a total mean body residue level of 14.2 µg/g DDT in goldfish after they were fed a diet containing 17.7 µg/g DDT for 192 days, which would indicate a concentration factor of 0.8 times.

Some ^{14}C-DDT that leached from the food into the water was observed by liquid-scintillation, and a mean ^{14}C-DDT water concentration of 0.065±0.007 µg/l (n=12) was determined. If this level was bioaccumulated 100,000 times, the highest level that could occur in the fish would be 6.5 µg/g, or about 10% of the total food-contributed residue. However, the total DDT

concentration measured in the water by gas-chromatographic analysis was not much greater where the fish were exposed to DDT-contaminated food only. If the control fish with a mean measured water concentration of 0.0029 µg/l DDT are compared to fish exposed only to DDT in the food (F) with a mean measured DDT water concentration of 0.0123 µg/l, the difference is only 0.009 µg/l, a possible body residue of 0.9 µg/g or about 1.5% of residues caused by DDT in the water leached from the food (assuming a 100,000 times magnification). Therefore, we believe that the contribution of labeled DDT through the water was negligible.

Our results indicate that DDT in the tissues decreased between 14 and 266 days. During the same period DDE and TDE residues increased. Grzenda et al. (1970) observed similar results for goldfish fed a DDT-contaminated diet for up to 192 days. We also observed in our test that the TDE residues were high in fish exposed to dietary DDT and were low in fish fed clean food. This indicates that most of the TDE in the fish fed a contaminated diet probably came from the food itself, which was quite high in TDE.

The clams metabolized DDT almost entirely to TDE and produced little DDE. Some discrepancy was observed in the amounts of TDE produced between exposures. Although all clam DDT exposures were held at the same temperature (20° C), clams for exposures 1 and 4 were collected in the fall and exposed in the spring, whereas clams for exposures 2 and 3 were collected in the spring and fall and exposed in the fall and winter. More clam metabolism would naturally occur in the spring than in the winter, and a seasonally controlled mechanism might be responsible for metabolite differences among the DDT-exposed clams. Another possible explanation is that differences could have been caused by different ratios of clam species present. No check was made as to the relative frequency of each species, although the genus Lampsilis appeared to be the one most prevalent. The 1 µg/g DDT found in the control fish was probably caused by the presence of 0.7 µg/g DDT in the first batch of clean clam tissue fed to the fish. This explanation is likely since the use of this clam food and the residue peak in the control fish terminated at about the same time.

The proportion of TDE and DDE produced appears to vary with the type of organism exposed. Rats convert most DDT to TDE, whereas humans usually metabolize DDT

to DDE (O'Brien, 1967). Mollusks, as observed in this test and by Cooke and Pollard (1973), metabolize DDT mainly to TDE, whereas fish metabolize DDT essentially to DDE (Priester, 1965; Johnson and Pecor, 1969; Reinert and Bergman, 1974). Ferguson et al. (1967), however, observed almost equal amounts of DDT, DDE, and TDE in several pooled samples of resistent mosquitofish (Gambusia affinis). Therefore, it is apparent that some fish can also readily produce large amounts of TDE.

Some breakdown of DDT to TDE may have occurred in our test when the clam tissue was ovendried or when it was in frozen storage. Breakdown of DDT to TDE in frozen storage was demonstrated by French and Jefferies (1971). Our clam tissue, however, was used within 60 days, so post-mortem breakdown is believed to have been negligible. Ovendrying also is not believed to have caused much DDT breakdown. Smith et al. (1973) found no large change in total TDE after fish steaks were baked at 177° C, and Metcalf (1955) stated that pure DDT is stable up to 195° C.

Lipid values could be correlated with residue values, an indication that DDT uptake was influenced by the lipid content of the fish. DDT-residue levels declined rapidly during the spawning period. Lipid content of the fish also decreased at this time in fish exposed to the 2.0 µg/l DDT water concentration whether they were fed clean or DDT-contaminated food. This is probably a fairly common occurrence. Reinert and Bergman (1974) observed that DDT residues were redistributed in the tissues of spawning-run fish and that this redistribution was closely related to a general decrease in the amount of fish oil.

The presence of DDT in the diet significantly reduced the probability of survival for exposed fish. Two definite periods of death were observed, the early larval stage up to 73 days of age and the spawning period, when highly colored males were the most sensitive. Fish that died at the spawning period were in relatively poor condition and did not feed. They probably used their fat reserves, thereby causing a release of stored DDT into the blood where the DDT could become toxic. Holden (1962) stated that fish in poor condition or with low fat content were more

susceptible to DDT toxicity. Our results support Holden's statement in that fish that died had predominantly lower lipid values than live fish sampled at the same time. Redistribution of DDT to the brain during weight loss with resultant deaths has been observed by some authors. Dale et al. (1962) observed high brain levels of DDT in rats that were starved after being fed DDT. Transfer of DDT to brains of birds and some resultant deaths were observed by Bernard (1966), Ecobichon and Saschenbrecker (1969), and Van Velzen et al. (1972). Redistribution of DDT and fat depletion in salmon and trout were observed during the spawning run by Holden (1962) and Reinert and Bergman (1974) and in the laboratory in starved rainbow trout by Grant and Schoettger (1972). Desaiah et al. (1975) analyzed fish sampled at 56, 118, 225, and 266 days during our toxicity test and found that partial chronic exposure to DDT significantly inhibited mitochondrial Mg^{2+} ATPase activity in the brain tissue of the fathead minnows.

In regard to toxicity in relation to the high concentrations of TDE in the food, the oral toxicity of TDE to rats (U.S. Environmental Protection Agency, 1972) is about 1/3 that of DDE. In bluegills, however, the static acute data of Cope (1965) and Mayer (personal communication) indicate that TDE is more toxic than DDE. Probably the relative toxicity of each varies with the specific organism exposed. Both DDE and TDE, however, appear to be less toxic than DDT (Oettingen and Sharpless, 1946; Rudd and Genelly, 1956; O'Brien, 1967; Moyle and Skrypek, 1969). Therefore, even though our clam tissue had high levels of TDE, our mortality results present a more accurate example of the DDT effect in a normal aquatic food chain than if we had used the more toxic unmetabolized DDT in a dry food mix.

To estimate the relative contribution of total DDT from the food to DDT from the water in regard to its effect on mortality the following model was assumed: the log of the total dose that a fish can tolerate has a normal distribution which can be approximated closely by a logistic distribution. Thus we have that (1) $\ln(P/1-P) = \gamma+\beta\ln x$, where x is the total dose delivered to the fish and P is the probability of death given a dose at x. It was further assumed that the water and food DDT act on the fish in a similar manner and thus x is proportional to the sum of water and food exposure and is expressed as (2) $x=\theta\ (x_1+\phi x_2)$, where θ is a magnification and absorption

factor in the target organ, x_1=concentration of DDT in the water, x_2=1 if DDT is in the food, zero if otherwise, and ρ is the unknown amount of DDT ingested from the food. Substituting the value for x into equation (1) we have that (3) $\ln(P/1-P) = \gamma+\beta\ln(\theta(x_1+\rho x_2)) = \alpha+\beta\ln(x_1=\rho x_2)$, where $\alpha=\gamma+\beta\ln\theta$ is a combination of parameters that is estimatable from the data. The probability of death given a target dose of x may be estimated from our data utilizing Abbott's formula from the relationship Ps=(1-Po) (1-P), where Po is the probability of death of a control fish. Thus P=1-Ps/(1-Po) is an estimated probability of death due to DDT, where Ps and 1-Po are taken as the average values in Table 5. Table 15 shows these estimated P values, the actual mean measured DDT water concentrations in duplicated exposure chambers, and the values of the estimated parameters obtained using non-linear least square estimation. The value $e^{-\alpha/\beta}$ is an estimate of LC50 caused by total exposure. It is estimated as 1.4596 µg/l in DDT water with no DDT in the food, or 0.9270 µg/l in DDT water with 45.6 µg/g DDT in the food. The amount of DDT in the food delivered to the target organism in this study is estimated as 0.5325 units in water. Thus the percentage of DDT in the fish from DDT in the contaminated food in the 0.5 and 2.0 µg/l DDT water exposures is estimated as 58.8 and 26.4, respectively. These estimates are very close to the measured percentages determined by liquid-scintillation analyses as shown in Table 4. Figure 9 shows the relationship between probability of death and DDT in the water with and without DDT in the food. At low DDT water concentrations the importance of the DDT-contaminated food is greater, and as DDT water concentration increases the importance of the food effect decreases. Dunnett's procedure (Steel and Torrie, 1960) was used to compare the combined duplicate test exposure mean probability of survival with that in the controls. With P=0.05, reduction in the probability of survival was significant only at the 2.0 W and 2.0 W + F exposures. By back calculation it is estimated that reduction in the probability of survival to 64.5% (35.5% mortality) is necessary to be significantly different. From Figure 9 it can be seen that this mortality level would fall at about 0.425 µg/l DDT in the water plus DDT in the food and at 0.925 µg/l DDT in the water without DDT in the food.

Our mortality data indicate that the presence of dietary DDT is more important when DDT water concentrations are low. A similar situation in nature could easily occur if food organisms built up tissue residues during pesticide

TABLE 15. ESTIMATED P VALUES, AVERAGE MEASURED DDT WATER CONCENTRATIONS, AND VALUES FOR ESTIMATED PARAMETERS FROM NON-LINEAR LEAST SOUARE ESTIMATION FOR FISH EXPOSED TO VARIOUS TEST CONDITIONS

P Estimated probability of death caused by DDT	ln(P/1-P)	X_1 Average measured DDT in the water (µg/l)	X_2 (0 if no DDT in food) (1 if DDT in food)
0.0598	-2.7550	0.3517	0
0.4680	-0.1280	1.5285	0
0.1489	-1.7430	0.0123	1
0.1685	-1.5960	0.3740	1
0.7534	1.1170	1.4839	1

$$\ln(P/1-P) = \alpha + \beta \ln(X_1 + \rho X_2)$$

Parameters	Estimates of parameters	Standard deviation of estimates
α	-0.7661	0.3802
β	2.0261	0.4313
ρ	0.5325	0.1946

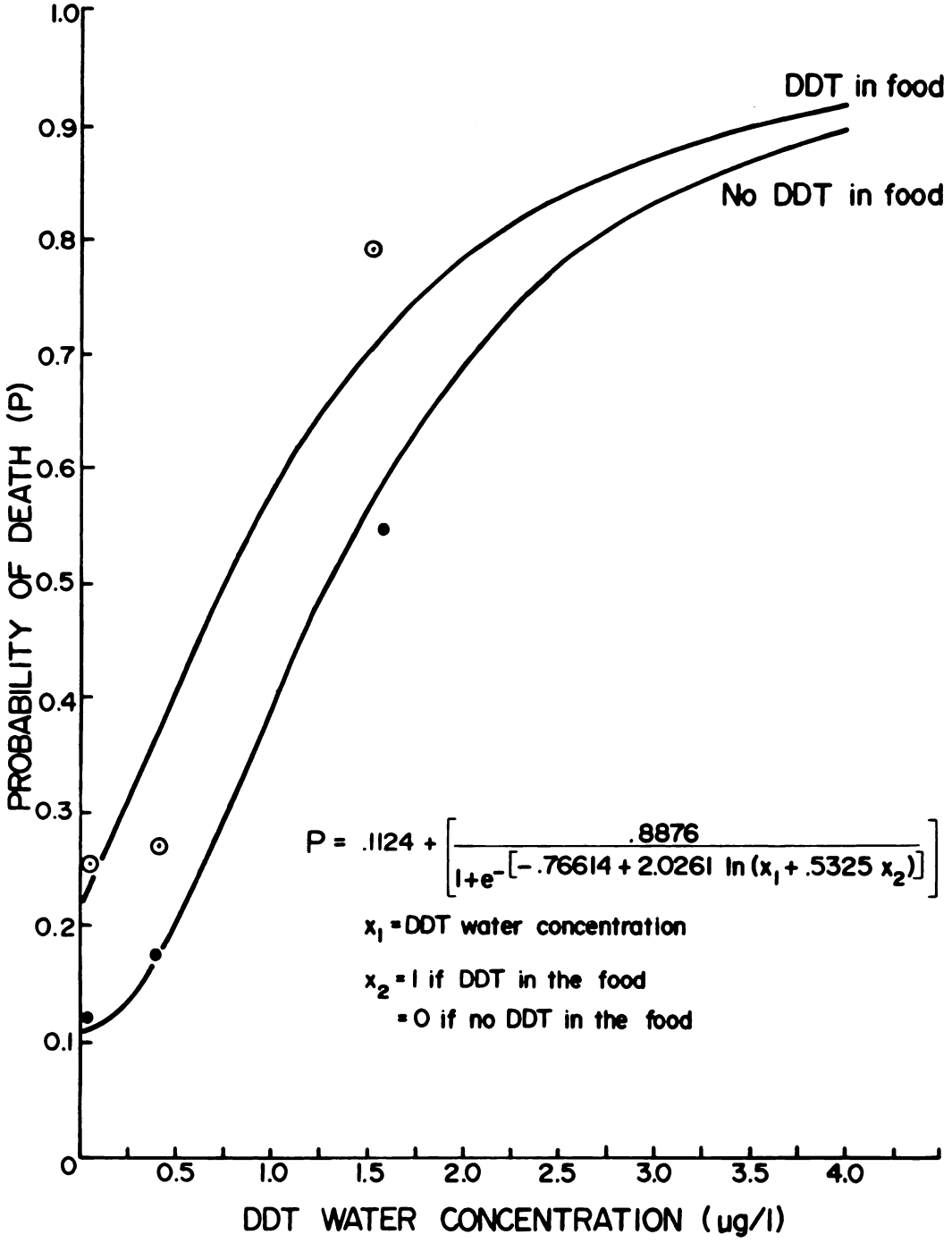

Figure 9. Estimated probability of death for fish exposed to DDT at various water concentrations and fed clean or DDT-contaminated food.

contamination and then were eaten by predators that entered the area after pesticide water concentrations had moderated, or if several successive food-chain accumulations occurred. Dietary DDT exposure is important at relatively higher water concentrations, although to a lesser degree than water exposure. This view is also held by other investigators, such as Grzenda et al. (1970), who stated that apparent DDT biological magnification by the food chain may not be as significant as the length of time fish are exposed to pesticide residues in the water; Reinert and Bergman (1974), who stated that rapid accumulation of DDT residues in coho salmon was probably related in part to an increase in the intake of DDT-contaminated alewifes; and Macek and Korn (1970), who demonstrated that dietary DDT can be very important, especially when compared with very low DDT water concentrations such as are found in Lake Michigan.

Embryo hatchability was reduced significantly only when parent fish were exposed to DDT at 2.0 µg/l in the water and fed clean food. However, since DDT tissue residues were twice as high in embryos from fish exposed to DDT both in the diet and at 2.0 µg/l in the water, the significant reduction of hatchability in the former group and not in the latter may have been the result of adult fathead minnow variability. Residues caused by dietary DDT were additive for all embryos from parent fish exposed to DDT in water and food. Huisman et al. (1971) reported that high residues of DDT, DDE, and TDE were accompanied by low fertility in pike. Kleinert and Degurse (1973) demonstrated a strong correlation between DDT content of embryos and larvae from walleyes in Wisconsin lakes, but could not associate the presence of DDT with success of the hatch. Burdick et al. (1972) demonstrated loss of brown trout and brook trout larvae hatched from eggs taken from female fish fed dietary DDT at different concentrations and durations of time. They also confirmed DDT as the cause of 100% mortality of larvae reared from eggs from lake trout fed 6 µg of DDT per gram of body weight per week. Average DDT egg residues less DDE were 7.61 and 11.92 µg/g for 2 separate years. In our test mean total DDT embryo residues less DDE were 4.31 µg/g from fish fed dietary DDT and 9.55 µg/g from fish fed dietary DDT and also exposed to DDT in the water. Lack of 100% larval mortality at these levels is probably explained by species variability, as fathead minnows are generally less susceptible to DDT than salmonids. Larvae from fish exposed at 2.0 W and 2.0 W + F reared at the same water concentration as the parent fish experienced 100% mortality within 5 days. Part of this

mortality may have been caused by DDT sorbed from the water, as larvae from these two parental groups transferred to control water for 30 days demonstrated an average of only 59% mortality among those from parents exposed to DDT in the water only and greater than 81% mortality among those from parent fish exposed to DDT in both the water and diet, an almost twofold higher mortality. These mortality data agree closely with embryo residue results that indicated almost twice as much DDT residue (40.9 vs. 24.0 µg/g) in embryos from parent fish exposed to DDT in both water and diet. Although residue levels were high, the larvae could either readily eliminate the DDT or dilute the residues through growth if placed in clean water. After 30 days in clean water these larvae, if they survived, had residues no greater than those in control larvae.

Total DDT residues were higher at all life stages whenever parent fish were exposed to DDT in both the water and diet than for water exposure alone. Even though DDT concentration may be greater from the water, presence of DDT also in the diet caused higher tissue residues and death rates than a corresponding water exposure alone. Death rates were not significant for larvae exposed to dietary DDT at 30 and 60 days, perhaps because some larvae could not adapt to clam tissue when active feeding commenced, and deaths occurred among the control fish by starvation. This was reflected in a mean mortality of 34% among control larvae at 30 days.

Results in the elimination phase of our study were similar to those observed by other authors. Grzenda et al. (1970) demonstrated a 50% elimination of DDT in goldfish by 29 days after exposure to dietary DDT for 192 days. Gakstatter and Weiss (1967) observed less than 50% DDT elimination after 32 days of recovery for bluegills that had been exposed to 0.03 mg/l DDT in the water for 5-19 hr. Buhler et al. (1969) observed an elimination of 45-68% of absorbed DDT in 35 days for chinook salmon and 19-35% elimination for coho salmon within a similar time period. Macek et al. (1970) predicted, from their results, a 50% elimination of total body DDT and dieldrin in rainbow trout within 160 and 40 days, respectively, after dietary exposure for 140 days. This elimination of DDT appears rather long, but it is most likely influenced by species variability. In our study almost all the eliminated DDT came from the DDT attributed to a dietary

source, an indication of preferential elimination. We do not believe that this selectivity was caused by metabolite differences, because the DDT, DDE, and TDE residues in fish that had been exposed to DDT only in the water essentially did not change. As simple kinetics will not explain these observations, unknown physiological factors are important, perhaps related to the different routes of entry.

We have demonstrated that even though DDT uptake may be faster from a water source, the presence of DDT in the food can cause higher tissue residues and a significant increase in mortality. The residues from dietary exposure were not as large as those observed for water exposure, but nevertheless the food chain must be considered an important component. A "just safe" water concentration or maximum acceptable toxicant concentration (MATC) determined from mortality results would be about 0.9 µg/l DDT for water exposure alone and about 0.4 µg/l DDT with the added presence of dietary DDT. This increase in toxicity is greater than 50%. Application factors as defined by Mount and Stephan (1967), using a 96-hr TL50 value of 48 µg/l DDT demonstrated in one of our acute fathead minnow toxicity tests and the maximum acceptable toxicant concentrations above, would be 0.0188 or 1/53 for fish exposed to DDT in the water alone and 0.0083 or 1/120 for fish exposed to DDT both in the water and diet. Consequently, food as well as water sources of exposure to certain materials must be considered when toxicity tests are designed or the conclusions drawn from such tests are evaluated.

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APPENDIX

RECOMMENDED BIOASSAY PROCEDURE FOR FATHEAD MINNOW PIMEPHALES PROMELAS RAFINESQUE CHRONIC TESTS

April, 1971

(Revised January, 1972)

by

Environmental Research Laboratory-Duluth
(formerly the National Water Quality Laboratory)
Duluth, Minnesota 55804

ENVIRONMENTAL RESEARCH LABORATORY-DULUTH
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RECOMMENDED BIOASSAY PROCEDURES

Preface

Recommended Bioassay Procedures are established by the approval of both the Committee on Aquatic Bioassays and the Director of the National Water Quality Laboratory. The main reasons for establishing them are: (1) to permit direct comparison of test results, (2) to encourage the use of the best procedures available, and (3) to encourage uniformity. These procedures should be used by National Water Quality Laboratory personnel whenever possible, unless there is a good reason for using some other procedure.

Recommended Bioassay Procedures consider the basic elements that are believed to be important in obtaining reliable and reproducible results in laboratory bioassays. An attempt has been made to adopt the best acceptable procedures based on current evidence and opinion, although it is recognized that alternative procedures may be adequate. Improvements in the procedures are being considered and tested, and revisions will be made when necessary. Comments and suggestions are encouraged.

Director, National Water Quality Lab, (NWQL)

Committee on Aquatic Bioassays, NWQL

Recommended Bioassay Procedure for

Fathead Minnow Pimephales promelas Rafinesque Chronic Tests

April, 1971

(Revised January, 1972)

A. Physical system

1. Diluter: Proportional diluters (Mount and Brungs, 1967) should be employed for all long-term exposures. Check the operation of the diluter daily, either directly or through measurement of toxicant concentrations. A minimum of five toxicant concentrations and one control should be used for each test with a dilution factor of not less than 0.30. An automatically triggered emergency aeration and alarm system must be installed to alert staff in case of diluter, temperature control or water supply failure.

2. Toxicant mixing: A container to promote mixing of toxicant bearing and w-cell water should be used between diluter and tanks for each concentration. Separate delivery tubes should run from this container to each duplicate tank. Check at least once every month to see that the intended amounts of water are going to each duplicate tank or chamber.

3. Tank: Two arrangements of test tanks (glass, or stainless steel with glass ends) can be utilized:

   a. Duplicate spawning tanks measuring 1 x 1 x 3 ft. long with a one sq. ft. portion at one end screened off and divided in half for the progeny. Test water is to be delivered separately to the larval and spawning chambers of each tank, with about one-third the water volume going to the former chamber as to the latter.

   b. Duplicate spawning tanks measuring 1 x 1 x 2 ft. long with a separate duplicate progeny tank for each spawning tank. The larval tank for each spawning tank should be a minimum of 1 cu. ft. dimensionally and divided to form two separate larval chambers with separate standpipes, or separate 1/2 sq. ft. tanks may be used. Test water is to be supplied by delivery tubes from the mixing cells described in Step 2 above.

   Test water depth in tanks and chambers for both a & b above should be 6 inches.

4. Flow rate: The flow rate to each chamber (larval or adult) should be equal to 6 to 10 tank volumes/24 hr.

5. Aeration: Total dissolved oxygen levels should never be allowed to drop below 60% of saturation, and flow rates must be increased if oxygen levels do drop below 60%. As a first alternative flow rates can be increased above those specified in A.4. Only aerate (with oil free air) if testing a non-volatile toxic agent, and then as a last resort to maintain dissolved oxygen at 60% of saturation.

6. Cleaning: All adult tanks, and larvae tanks and chambers after larvae swim-up, must be siphoned a minimum of 2 times weekly and brushed or scraped when algal or fungus growth becomes excessive.

7. Spawning substrate: Use spawning substrates made from inverted cement and asbestos halved, 3-inch ID drain tile, or the equivalent, each of these being 3 inches long.

8. Egg cup: Egg incubation cups are made from either 3-inch sections of 2-inch OD (1 1/2-inch ID) polyethylene water hose or 4-oz., 2-inch OD round glass jars with the bottoms cut off. One end of the jar or hose sections is covered with stainless steel or nylon screen (with a minimum of 40 meshes per inch). Cups are oscillated in the test water by means of a rocker arm apparatus driven by a 2 r.p.m. electric motor (Mount, 1968). The vertical-travel distance of the cups should be 1 to 1 1/2 inches.

9. Light: The lights used should simulate sunlight as nearly as possible. A combination of Durotest (Optima FS)[1,2] and wide spectrum Grow-lux[3] fluorescent tubes has proved satisfactory at the NWQL.

10. Photoperiod: The photoperiods to be used (Appendix A) simulate the dawn to dusk times of Evansville, Indiana. Adjustments in day-length are to be made on the first and fifteenth day of every Evansville test month. The table is arranged so that adjustments need be made only in the dusk times. Regardless of the actual date that the experiment is started, the Evansville test photoperiod should be adjusted so that the mean or estimated hatching date of the fish used to start the experiment corresponds to the Evansville test day-length for December first. Also, the dawn and dusk times listed in the table need not correspond to the actual times where the experiment is being conducted. To illustrate these points, an experiment started with 5-day-old larvae in Duluth, Minnesota, on August 28 (actual date), would require use of a December 5 Evansville test photoperiod, and the lights could go on anytime on that day just so long as they remained on for 10 hours and 45 minutes. Ten days later (Sept. 7 actual date, Dec. 15 Evansville test date) the day-length

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[1] Mention of trade names does not constitute endorsement.

[2] Duro-Test, Inc., Hammond, Ind.

[3] Sylvania, Inc., New York, N. Y.

would be changed to 10 hours and 30 minutes. Gradual changes in light intensity at dawn and dusk (Drummond and Dawson, 1970), if desired, should be included within the day-lengths shown, and should not last for more than 1/2 hour from full on to full off and vice versa.

11. Temperature: Temperature should not deviate instantaneously from 25° C by more than 2° C and should not remain outside the range of 24 to 26° C for more than 48 hours at a time. Temperature should be recorded continuously.

12. Disturbance: Adults and larvae should be shielded from disturbances such as people continually walking past the chambers, or from extraneous lights that might alter the intended photoperiod.

13. Construction materials: Construction materials which contact the diluent water should not contain leachable substances and should not sorb significant amounts of substances from the water. Stainless steel is probably the preferred construction material. Glass absorbs some trace organics significantly. Rubber should not be used. Plastic containing fillers, additives, stabilizers, plasticizers, etc., should not be used. Teflon, nylon, and their equivalents should not contain leachable materials and should not sorb significant amounts of most substances. Unplasticized polyethylene and polypropylene should not contain leachable substances, but may sorb very significant amounts of trace organic compounds.

14. Water: The water used should be from a well or spring if at all possible, or alternatively from a surface water source. Only as a last resort should water from a chlorinated municipal water supply be used. If it is thought that the water supply could be conceivably contaminated with fish pathogens, the water should be passed through an ultraviolet or similar sterilizer immediately before it enters the test system.

B. Biological system

1. Test animals: If possible, use stocks of fathead minnows from the National Water Quality Laboratory in Duluth, Minnesota or the Fish Toxicology Laboratory in Newtown, Ohio. Groups of starting fish should contain a mixture of approximately equal numbers of eggs or larvae from at least three different females. Set aside enough eggs or larvae at the start of the test to supply an adequate number of fish for the acute mortality bioassays used in determining application factors.

2. Beginning test: In beginning the test, distribute 40 to 50 eggs or 1 to 5-day-old larvae per duplicate tank using a stratified random assignment (see D.3). All acute mortality tests should be conducted when the fish are 2 to 3 months old. If eggs or 1 to 5-day-old larvae are not available, fish up to 30 days of age may be used to start the test. If fish between 20 and 60 days old are used, the exposure should be designated a partial chronic test. Extra test animals may be added at the beginning so that fish can be removed periodically for special examinations (see B.12.) or for residue analysis (see C.4.).

3. Food: Feed the fish a frozen trout food (e.g., Oregon Moist). A minimum of once daily fish should be fed ad libitum the largest pellet they will take. Diets should be supplemented weekly with live or frozen-live food (e.g., Daphnia, chopped earthworms, fresh or frozen brine shrimp, etc.). Larvae should be fed a fine trout starter a minimum of 2 times daily, ad libitum; one feeding each day of live young zooplankton from mixed cultures of small copepods, rotifers, and protozoans is highly recommended. Live food is especially important when larvae are just beginning to feed, or about 8 to 10 days after egg deposition. Each batch of food should be checked for pesticides (including DDT, TDE, dieldrin, lindane, methoxychlor, endrin, aldrin, BHC, chlordane, toxaphene, 2,4-D, and PCBs), and the kinds and amounts should be reported to the project officer or recorded.

4. Disease: Handle disease outbreaks according to their nature, with all tanks receiving the same treatment whether there seems to be sick fish in all of them or not. The frequency of treatment should be held to a minimum.

5. Measuring fish: Measure total lengths of all starting fish at 30 and 60 days by the photographic method used by McKim and Benoit (1971). Larvae or juveniles are transferred to a glass box containing 1 inch of test water. Fish should be moved to and from this box in a water-filled container, rather than by netting them. The glass box is placed on a translucent millimeter grid over a fluorescent light platform to provide background illumination. Photos are then taken of the fish over

the millimeter grid and are enlarged into 8 by 10 inch prints. The length of each fish is subsequently determined by comparing it to the grid. Keep lengths of discarded fish separate from those of fish that are to be kept.

6. Thinning: When the starting fish are sixty (± 1 or 2) days old, impartially reduce the number of surviving fish in each tank to 15. Obviously injured or crippled individuals may be discarded before the selection so long as the number is not reduced below 15; be sure to record the number of deformed fish discarded from each tank. As a last resort in obtaining 15 fish per tank, 1 or 2 fish may be selected for transfer from one duplicate to the other. Place five spawning tiles in each duplicate tank, separated fairly widely to reduce interactions between male fish guarding them. One should also be able to look under tiles from the end of the tanks. During the spawning period, sexually maturing males must be removed at weekly intervals so there are no more than four per tank. An effort should be made not to remove those males having well established territories under tiles where recent spawnings have occurred.

7. Removing eggs: Remove eggs from spawning tiles starting at 12:00 noon Evansville test time (Appendix A) each day. As indicated in Step A.9., the test time need not correspond to the actual time where the test is being conducted. Eggs are loosened from the spawning tiles and at the same time separated from one another by lightly placing a finger on the egg mass and moving it in a circular pattern with increasing pressure until the eggs being to roll. The groups of eggs should then be washed into separate, appropriately marked containers and subsequently handled (counted, selected for incubation, or discarded) as soon as possible after all eggs have been removed and the spawning tiles put back into the test tanks. All egg batches must be checked initially for different stages of development. If it is determined that there is more than one distinct stage of development present, then each stage must be considered as one spawning and handled separately as described in Step B.8.

8. Egg incubation and larval selection: Impartially select 50 unbroken eggs from spawnings of 50 eggs or more and place them in an egg incubator cup for determining viability and hatchability. Count the remaining eggs and discard them. Viability and hatchability determinations must be made on each spawning (>49 eggs) until the number of spawnings (>49 eggs) in each duplicate tank equals the

number of females in that tank. Subsequently, only eggs from every third spawning (>49 eggs) and none of those obtained on weekends need be set up to determine hatchability; however, weekend spawns must still be removed from tiles and the eggs counted. If unforseen problems are encountered in determining egg viability and hatchability, additional spawnings should be sampled before switching to the setting up of eggs from every third spawning. Every day record the live and dead eggs in the incubator cups, remove the dead ones, and clean the cup screens. Total numbers of eggs accounted for should always add up to within two of 50 or the entire batch is to be discarded. When larvae begin to hatch, generally after 4 to 6 days, they should not be handled again or removed from the egg-cups until all have hatched. Then, if enough are still alive, 40 of these are eligible to be transferred immediately to a larval test chamber. Those individuals selected out to bring the number kept to 40 should be chosen impartially. Entire egg-cup-groups not used for survival and growth studies should be counted and discarded.

9. Progeny transfer: Additional important information on hatchability and larval survival is to be gained by transferring control eggs immediately after spawning to concentrations where spawning is reduced or absent, or to where an affect is seen on survival of eggs or larvae, and by transferring eggs from these concentrations to the control tanks. One larval chamber in, or corresponding to, each adult tank should always be reserved for eggs produced in that tank.

10. Larval exposure: From early spawnings in each duplicate tank, use the larvae hatched in the egg incubator cups (Step B.8. above) for 30 or 60 day growth and survival exposures in the larval chambers. Plan ahead in setting up eggs for hatchability so that a new group of larvae is ready to be tested for 30 or 60 days as soon as possible after the previously tested group comes out of the larval chambers. Record mortalities, and measure total lengths of larvae at 30 and, if they are kept, 60 days post-hatch. At the time the larval test is terminated they should also be weighed. No fish (larvae, juveniles, or adults) should be fed within 24 hr's. of when they are to be weighed.

11. Parental termination: Parental fish testing should be terminated when, during the receding day-length photoperiod, a one week period passes in which no spawning occurs in any of the tanks. Measure total lengths and weights of parental fish; check sex and condition of gonads. The gonads of most parental fish will have begun to regress from the spawning condition, and thus the differences between the sexes will be less distinct now than previously. Males and females that are readily distinguishable from one another because of their external characteristics should be selected initially for determining how to differentiate between testes and ovaries. One of the more obvious external characteristics of females that have spawned is an extended, transparent anal canal (urogenital papilla). The gonads of both sexes will be located just ventral to the kidneys. The ovaries of the females at this time will appear transparent, but perhaps containing some yellow pigment, coarsely granular, and larger than testes. The testes of males will appear as slender, slightly milkly, and very finely granular strands. Fish must not be frozen before making these examinations.

12. Special examinations: Fish and eggs obtained from the test should be considered for physiological, biochemical, histological and other examinations which may indicate certain toxicant related effects.

13. Necessary data: Data that must be reported for each tank of a chronic test are:

    a. Number and individual total length of normal and deformed fish at 30 and 60 days; total length, weight and number of either sex, both normal and deformed, at end of test.

    b. Mortality during the test.

    c. Number of spawns and eggs.

    d. Hatchability.

    e. Fry survival, growth, and deformities.

C. Chemical system

1. Preparing a stock solution: If a toxicant cannot be introduced into the test water as is, a stock solution should be prepared by dissolving the toxicant in water or an organic solvent. Acetone has been the most widely used solvent, but dimethylformanide (DMF) and triethylene glycol may be preferred in many cases. If none of these solvents are acceptable, other water-miscible solvents such as methanol, ethanol, isopropanol, acetonitrile, dimethylacetamide (DMAC), 2-ethoxyethanol, glyme (dimethylether of ethylene glycol, diglyme (dimethyl ether of diethylene glycol) and propylene glycol should be considered. However, dimethyl sulfoxide (DMSO) should not be used if at all possible because of its biological properties.

   Problems of rate of solubilization or solubility limit should be solved by mechanical means if at all possible. Solvents, or as a last resort, surfactants, can be used for this purpose, only after they have been proven to be necessary in the actual test system. The suggested surfactant is p-tert-octylphenoxynonaethoxyethanol (p-1, 1, 3, 3-tetramethylbutylphenoxynonaethoxyethanol, OPE_{10}) (Triton X-100, a product of the Rohm and Haas Company, or equivalent).

   The use of solvents, surfactants, or other additives should be avoided whenever possible. If an additive is necessary, reagent grade or better should be used. The amount of an additive used should be kept to a minimum, but the calculated concentration of a solvent to which any test organisms are exposed must never exceed one one-thousandth of the 96-hr. TL50 for test species under the test conditions and must never exceed one gram per liter of water. The calculated concentration of surfactant or other additive to which any test organisms are exposed must never exceed one-twentieth of the concentration of the toxicant and must never exceed one-tenth gram per liter of water. If any additive is used, two sets of controls must be used, one exposed to no additives and one exposed to the highest level of additives to which any other organisms in the test are exposed.

2. Measurement of toxicant concentration: As a minimum the concentration of toxicant must be measured in one tank at each toxicant concentration every week for each set of duplicate tanks, alternating tanks at each concentration from week to week. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include any surface scum or material stirred up from the bottom or sides of the tank. Equivolume daily grab samples can be composited for a week if it has been shown that the results of the analysis are not affected by storage of the sample.

Enough grouped grab samples should be analyzed periodically throughout the test to determine whether or not the concentration of toxicant is reasonably constant from day to day in one tank and from one tank to its duplicate. If not, enough samples must be analyzed weekly throughout the test to show the variability of the toxicant concentration.

3. Measurement of other variables: Temperature must be recorded continuously (see A.10.).

   Dissolved oxygen must be measured in the tanks daily, at least five days a week on an alternating basis, so that each tank is analyzed once each week. However, if the toxicant or an additive causes a depression in dissolved oxygen, the toxicant concentration with the lowest dissolved oxygen concentration must be analyzed daily in addition to the above requirement.

   A control and one test concentration must be analyzed weekly for pH, alkalinity, hardness, acidity, and conductance or more often, if necessary, to show the variability in the test water. However, if any of these characteristics are affected by the toxicant the tanks must be analyzed for that characteristic daily, at least five days a week, on an alternating basis so that each tank is analyzed once every other week.

   At a minimum, the test water must be analyzed at the beginning and near the middle of the test for calcium, magnesium, sodium, potassium, chloride, sulfate, total solids, and total dissolved solids.

4. Residue analysis: When possible and deemed necessary, mature fish, and possibly eggs, larvae, and juveniles, obtained from the test, should be analyzed for toxicant residues. For fish, muscle should be analyzed, and gill, blood, brain, liver, bone, kidney, GI tract, gonad, and skin should be considered for analysis. Analyses of whole organisms may be done in addition to, but should not be done in place of, analyses of individual tissues, especially muscle.

5. Methods: When they will provide the desired information with acceptable precision and accuracy, methods described in Methods for Chemical Analysis of Water and Wastes (EPA, 1971) should be used unless there is another method which requires much less time and can provide the desired information with the same or better precision and accuracy. At a minimum, accuracy should be measured using the method of known additions for all analytical methods for toxicants. If available, reference samples should be analyzed periodically for each analytical method.

D. Statistics

1. Duplicates: Use true duplicates for each level of toxic agent, i.e., no water connections between duplicate tanks.

2. Distribution of tanks: The tanks should be assigned to locations by stratified random assignment (random assignment of one tank for each level of toxic agent in a row followed by random assignment of the second tank for each level of toxic agent in another or an extension of the same row).

3. Distribution of test organisms: The test organisms should be assigned to tanks by stratified random assignment (random assignment of one test organism to each tank, random assignment of a second test organism to each tank, etc.).

E. Miscellaneous

1. Additional information: All routine bioassay flow through methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) should be followed as described in Standard Methods for the Examination of Water and Wastewater, (American Public Health Association, 1971), or information requested from appropriate persons at Duluth or Newtown.

2. Acknowledgments: These procedures for the fathead minnow were compiled by John Eaton for the Committee on Aquatic Bioassays. The participating members of this committee are: Robert Andrew, John Arthur, Duane Benoit, Gerald Bouck, William Brungs, Gary Chapman, John Eaton, John Hale, Kenneth Hokanson, James McKim, Quentin Pickering, Wesley Smith, Charles Stephan, and James Tucker.

3. References: For additional information concerning flow through bioassays with fathead minnows, the following references are listed:

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Approved by the Committee
on Aquatic Bioassays, NWQL

Approved by the Director, NWQL

Appendix A

Test (Evansville, Indiana) Photoperiod

For Fathead Minnow Chronic

Dawn to Dusk Time	Date	Day-length (hour and minute)	
6:00 - 4:45)	DEC. 1	10:45)	
6:00 - 4:30)	15	10:30)	
		)	
6:00 - 4:30)	JAN. 1	10:30)	
6:00 - 4:45)	15	10:45)	
		)	
6:00 - 5:15)	FEB. 1	11:15)	5-month pre-
6:00 - 5:45)	15	11:45)	spawning growth
		)	period
6:00 - 6:15)	MAR. 1	12:15)	
6:00 - 7:00)	15	13:00)	
		)	
6:00 - 7:30)	APR. 1	13:30)	
6:00 - 8:15)	15	14:15)	
6:00 - 8:45)	MAY 1	14:45)	
6:00 - 9:15)	15	15:15)	
		)	
6:00 - 9:30)	JUNE 1	15:30)	
6:00 - 9:45)	15	15:45)	4-month spawning
		)	period
6:00 - 9:45)	JULY 1	15:45)	
6:00 - 9:30)	15	15:30)	
		)	
6:00 - 9:00)	AUG. 1	15:00)	
6:00 - 8:30)	15	14:30)	
6:00 - 8:00)	SEPT. 1	14:00)	
6:00 - 7:30)	15	13:30)	
		)	
6:00 - 6:45)	OCT. 1	12:45)	post spawning period
6:00 - 6:15)	15	12:15)	
		)	
6:00 - 5:30)	NOV. 1	11:30)	
6:00 - 5:00)	15	11:00)	

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

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15. SUPPLEMENTARY NOTES

16. ABSTRACT

Fathead minnows (Pimephales promelas) were exposed during a partial chronic toxicity test to two DDT concentrations in the water, one in the diet, and combinations of water and diet for 266 days through a reproductive period of their life cycle. Tissue-residue analyses were performed on test fish at preset intervals throughout the exposure and also on embryos, larvae at hatch, and 30- and 60-day progeny. The contribution of DDT from each source was monitored with gas-chromatography and liquid-scintillation techniques. The diet was clams that had accumulated 14C-DDT when exposed at a DDT water concentration similar to that in the high fish exposure.

Higher total DDT tissue residues were accumulated from the water than from the diet. Residues contributed by dietary DDT were additive to those from the water. Mean concentration factors were 1.2 times from the diet and 100,000 times from the water. Mortality was higher in fish exposed to DDT in both water and diet than in fish exposed to only one or the other of these sources. DDT in the diet significantly reduced the probability of survival of the test fish (P=0.025). Estimated maximum acceptable toxicant concentrations for DDT are 0.9 µg/l for fish exposed to DDT in the water only or 0.4 µg/l for fish exposed to DDT in both water and diet.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS		b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Bioassay*	Insecticides	Aquatic life	6A
Aquatic animals	Food chain	Chlorinated hydrocarbons	6C
Minnows*	Toxicity	Bioaccumulation	6F
Clams	Carbon 14	Tissue Residues	7B
Pesticides*			7C
DDT*			7E
Freshwater fishes			

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